Synopsis of the proposed PhD work entitled

Protein expression profiling of *Labeo rohita* infected with *Edwardsiella tarda*

For the award of degree of Doctor of Philosophy

In Applied Zoology

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May, 2024

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1. Introduction

In developing countries, aquaculture plays a crucial role in food security and livelihoods. The current market value of aquaculture output in 2018 was USD 401 billion, with a significant contribution estimated at USD 250 billion from aquaculture production (SOFIA, 2020). In India, the intake of animal dietary proteins is constantly on the rise, and aquaculture has shown an exceptional growth rate in combating the sustainable economy in the food sector. Indian aquaculture has been second rank in terms of global aquaculture production (Dinakaran, 2017). Aquaculture production has been increasing substantially over the last decades in India, contributing to a significant proportion of freshwater fish. Among freshwater aquaculture, carps contributes between 70% and 75% of total production (Mishra et al., 2017, FAO, 2018). Rohu (*Labeo rohita*) is one of most widely cultivated species at a market rate among the Indian major carps in the country. Of the global production of freshwater carp, rohu accounts for ~3.7% of world aquaculture (SOFIA, 2020). Rapid expansion of aquaculture and recent innovative technologies in culture endorse higher yields. Consequently, fish have substantial exposure to both pathogenic and nonpathogenic modes of microbes shortly after spawning from their protective chorions in water. Exposure of pathogenic modes of outbreaks, such as various opportunistic pathogens has caused devastating losses and has led to safety concerns in aquaculture.

Omics is an innovative research method used in a specific biological sample for tissue-specific genes, transcripts, proteins, lipids, as well as other biomolecules in an accurate and untargeted manner to better understand an organism's physiological functions. In all organisms, proteins are abundant and their importance as transporters, catalysts and messengers in most biological processes. Proteins are the major macromolecules made of amino acids. Proteomics-based techniques used in various fields of research, such as identification of molecular diagnostic markers, vaccine production candidates, understanding of pathogenicity mechanisms, changing patterns of expression in response to different signals, and analysis of protein pathways in diseases (Aslam et al., 2017). The major aspect of proteome research is the concurrent identification and determination of concentration ratios of proteins expressed in cells or tissues of different states. The changes in proteome expression under complex field

situations may disclose which gene products or proteins are most interesting to investigate. Protein labelling through a non-gel based technique such as isobaric tag for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT) have developed for quantitative proteomic in MS based technique. The simultaneous identification and measurement of protein expression concentration ratios in cells or tissues of various stages is a key component of proteome research. The incidence of infections is associated to diverse protein interactions between a host and a microbe. It is important to understand the Protein - Protein Interactions (PPIs) pathway of the disease mechanism. The PPI network – may deepen the knowledge of the various diseases and enhance the prevention programs of aquaculture species. Available in-depth protein databases for rohu and interactions between protein groups and proteome-wide linkages could be easily recognized for better understanding of cellular biochemistry and physiology of an organism (Hao et al., 2014). While investigating the pathophysiology mechanism, it is still important to recognize and quantify both proteins and their function within a cell, tissue or organism that is crucial for early diagnosis, prognosis and disease development. Targeted proteomics involve monitoring of precursor and transition list of MS, focused on detecting only the peptide/s of the selected biomarkers. Targets were important in aquaculture fields of clinical diagnosis kits and antibiotic resistance detection and any physiological changes that occurred during culture period is easily identified. Targets were important in aquaculture fields of clinical diagnosis kits and antibiotic resistance detection and any physiological changes that occurred during culture period is easily identified.

The prevalence of bacterial infections, the principle of disease detection and the controlled prospects for the development of freshwater aquaculture in India remained open to challenge. Proper understanding of the host-microbe encounters has therefore resulted in a clear development of various ways of preventing and treating infection-induced diseases. This study discusses the drawbacks of a label-free and label based proteomic technique in bacterial infected rohu tissues. In this context, the attempts to bring quantitative proteomics studies to address the issues associated with disease using the application of label and recently label free proteomics techniques for faster, cleaner and simpler way.

2. Review of literature

2.1. Indian aquaculture scenario

In the current scenario (2020), global aquaculture production reached 87.5 million tonnes of food fish, and among cultured fish production, 57.5 million tonnes represented finfish with the majority from 49.1 million tonnes of inland aquaculture (FAO, 2022). Currently, India is a top inland fisheries producer with 1.8 million tonnes (FAO, 2022). Indian aquaculture has registered excellent growth from a meagre level and reached about 1.03% of the National Gross Domestic Production (GDP) and 6.58% of the agricultural GDP (Rajeev and Bhandarkar, 2022). The main cultured fish species in India are Indian Major Carps (IMC), which include rohu (*Labeo rohita*), catla (*Catla catla*), mrigal (*Cirrhinus mrigala*), and exotic carps like, grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmicthys molitrix*), and common carp (*Cyprinus carpio*) and catfishes like Indian magur (*Clarius batrachus*), singhi (*Heteropneuestes fossilis*), pangas (*Pangassius* spp.) along with freshwater crustaceans like prawn (*Macrobrachium rosenbergii*). Freshwater prawn is cultured vary widely, which further accounts for aquaculture yield. The exotic catfish culture of *Pangasiandon hypophthalamus*, and Pacu (*Piaractus brachypomus*) are also increasing in these few years. Culturing Tilapia (*Oreochromis* spp.) and *Pangassius* spp in cages in inland water bodies such as lakes and reservoirs can provide scope for increasing fish productions. The main focus has been on the production of genetically improved tilapia (GIFT) for the market as a cheap source of proteins (De Jong, 2017). In the world, cyprinids, carps, and barbels, with a share of 18%, dominated the main group of species in the production of aquatic animals (FAO, 2022). Present capture and culture fish production reached 16.25 million tonnes providing a cheap source of protein and contributing to nutritional security (DAHD, 2023). There is a great scope for its expansion; however, the constant outbreak of diseases and epizootics is likely to be a significant bottleneck to improving production. Diseases in aquaculture account for 5-10% of the production cost (Sahoo et al., 2017). These infections are mainly of bacterial and parasitic origin, and while the presence of fungal pathogens and viruses in Indian aquaculture practices is rarely recorded.

2.2. Disease development process in aquaculture

Disease is a biological condition in which the normal functions of the body are disrupted due to changes in the body systems, and are usually manifested by differentiating symptoms and signs (Idowu et al., 2017). Healthy fish have sufficient resistance to disease, are able to adapt to appropriate environmental changes, and become resistant to disease. However, both primary and secondary stressors may contribute to changes in whole animal behavior, most likely by redirecting energy and other resources from one set of physiological processes to another, resulting in lower disease tolerance and increased pathology during infection (Vargas-Chacoff et al., 2016). The expansion of aquaculture farming through the introduction of new fish species and intensified practices has put tremendous pressure on sustainability. Like other production systems, sustainability in aquaculture is also dependent on the delicate balance between host, pathogen and environment. Aquaculture environments can emphasize the host, adapt to pathogenic factors and result in disease development. In aquatic ecosystems, disease prevention has become a major challenge due to the distinctive environment in which the pathogen frequently finds an opportunity once the health status of the host is compromised. Disease outbreaks in the Asian (China, India, and Vietnam) fisheries sector were estimated to account for > 30% of the overall production loss (Mohd-Aris et al., 2019).

2.2.1. Bacterial diseases in aquaculture

Disease is a major constraint for the growth of the aquaculture sector that can influence the socioeconomic development of many countries around the globe (Bagum et al., 2013, Mishra et al., 2017). Bacterial pathogens account for > 20% of overall outbreaks of disease in the Indian aquaculture sector according to global patterns (Mishra et al., 2017). Water quality is one of the crucial factors that can affect fish health status. Moreover, natural aquatic environment get contaminated due to anthropogenic activity and farming activities that lead to emergence of new pathogens. The infected aquatic animals present higher mortalities at warmer temperatures (Reverter et al., 2020). A wide range of bacterial pathogens have been associated with fish disease (Austin and Austin, 2016). More than hundred bacterial pathogens of fish and shellfish have been reported (Alderman and Hastings, 1998, Winton, 2001). Fish have intimate

contact with their environment, which can contain very high concentrations of numerous microflora. Many of these are saprophytic, most are pathogenic, and both are extremely capable of digesting and destroying fish tissue (Ellis, 2001). Bacteria are inevitable in the water environment; they can reproduce in large numbers, invade fish and spread the disease when reaching a suitable condition (Inglis et al., 1993). Pathogens originating from water can spread at a faster rate than those in terrestrial systems (McCallum et al., 2004). The severity of the disease is affected by various interrelated factors, such as bacterial virulence, the extent and degree of stress on the fish, the physiological state of the host, and the degree of genetic resistance inherent in particular fish populations (Cipriano and Bullock, 2001).

The balance between three major factors host, pathogen, and environment determines the state of health of aquatic species. Any alteration between these three major factors results in stressful conditions for the host. During disease conditions fish show erratic swimming, anorexia, loss of equilibrium, and disorganized swimming/sluggish movement (Soto et al., 2009, Ou et al., 2013). Behavior could provide external knowledge on the magnitude of changes on an individual basis for disease-related fish, as behavior incorporates both internal mechanisms of physiological and genetic networks (Hill et al., 2005).

2.2.1.1Major bacterial diseases in Indian freshwater fish

Numerous pathogenic species representing the mainstream of existing bacterial taxa have been associated with fish diseases, and only a relatively small number of pathogens are responsible for serious financial losses in cultivated fish worldwide. In India and developing countries, bacterial fish pathogens are very common and are one of the most difficult health problems to deal with at every stage from fry, fingerlings to adult stage causing morbidity and heavy mortality (Mishra et al., 2017). Some of the important bacterial diseases like motile aeromonad septicaemia caused by *Aeromonas hydrophila*, Edwardsiellosis caused by *Edwardsiella tarda*, Pseudomonas septicaemia caused by *Pseudomonas fluorescens* and *Pseudomonas putrefaciens*, flexibacteriosis caused by *Flexibacter columnar* are often reported in Indian carp culture (Mohanty and Sahoo, 2007).

2.2.1.2. Edwardsiellosis

The genus *Edwardsiella* is composed of three species, *E. tarda*, *E. ictaluri*, and *E. hoshinae* (Ewing et al., 1965, Hawke et al., 1981). It is a zoonotic opportunistic pathogen with a wide host range comprising reptiles, fish and mammals. *E. tarda* is a short, rod–shaped, motile, facultative anaerobic bacterium that measures about 2–3 μm in length and 1 μm in diameter. One of the important features of *E. tarda* is the ability to survive and replicate in various host cells. *E. tarda* able to bind and penetrating epithelial cells. In aquaculture edwardsiellosis, caused by *E. tarda* and *E. ictaluri* is a gram-negative bacterium of the Enterobacteriaceae family that can infect a wide range of freshwater and marine fish (Park et al., 2012) such as flounder, turbot, tongue sole, tilapia (Wang and Sun, 2015, Katharios et al., 2015, Zeng et al., 2017). The disease has appeared through *E. tarda* is also known as hepatonephritis in eel, and in channel catfish emphysematous putrefactive disease, or fish gangrene due to the foul smell of gas-filled pockets in the necrotic muscle. In warm water fish, first isolated in Japan by Hoshina (1962) and named *Paracolobacterium anguillimortiferum* was reexamined and identified as *E. tarda* by Wakabayashi and Egusa (1973) (Wakabayashi and Egusa, 1973). *E. tarda* was classified as systemic hemorrhagic septicemia with inflammation of the skin along with ulcer and necrosis of internal organs such as the liver, kidney, spleen and musculature in the disorder. It has been reported globally in important commercial fish species, including Japanese eel (*Anguilla japonica*), red sea bream (*Pagrus major*), yellowtail (*Seriola quinqueradiata*), channel catfish (*Ictalurus punctatus*), and turbot (*Scophthalmus maximus*). In the integrated farming, fish exposed to animal excreta and organic content is more prone for *E. tarda* infection. In the tilapia integration with pig farm has lead high organic content in water and found infection distribution of *E. tarda* was highest at external surface (Muratori et al., 2000).

2.3. Advancement in management of fish disease

In aquaculture, disease management and bringing down of cost associate with disease outbreaks has a challenge for large scale fish production. In most of the case, fish farmer has incorporated antibiotics, chemotherapeutic agents, pesticides, immunostimulant, and vaccine in dealing with pathogen to protect

production. Despite each strategy's benefits, the disadvantages overshadow the advantages in the long run. Indiscriminate use of antibiotic in aquaculture may have impacts on the environment and human health (Pinto et al., 2023, Samanidou and Evaggelopoulou, 2007). Vaccination also known as active immunity and prominent approaches that have been used for strengthening host immunity and defending fish from both existing and future infections. In bacterial infection, few vaccines have been developed for both marine and freshwater. Vaccines for fish are primarily based on inactivated pathogens combined with adjuvants and administered through immersion or injection (Irshath et al., 2023). In order to control aquatic disease, multiple fish vaccines are being used in worldwide, and currently as per USDA (United States Department of Agriculture) report, 77 types of fish vaccines against more than 22 bacterial and 6 viral pathogenic species available for treatment (Irshath et al., 2023, Tizard, 2021).

2.4. Omics Tools for in-depth fish immune response due to pathogens

In developing therapeutics, first needs to understand the fundamental biological system involved in infection, such as host-pathogen interactions and molecular regulatory networks (He et al., 2012). The novel biotechnological tools growing in aquaculture sector to meet biological constraints more emerged omics technologies, i.e., genomics, transcriptomics, proteomics, and metabolomics, have been used to improve in-depth knowledge of aquaculture production, health, safety and quality (Natnan et al., 2021a, Natnan et al., 2021b). In genetic and transcript level of omics involving expression of gene in identifying immune-related genes of the host, in response to diseases. While for protein and metabolite provide information on the biomarker discovery and in-depth immune pathway during infection (Natnan et al., 2021b). The systematic study exploring host-pathogen omics datasets will enable scientist to describe the complexity and characteristics of interactions, leading to identifying proper targets during infection caused pathogens. In developing new vaccine omics tools involved in targeting specific pathogenic components, including the use of recombinant genes and proteins.

2.4.1. Overview of omics technology used in rohu

Before whole genome study, part of the genes or transcript were characterized in rohu. In *A. hydrophila* infection, a resistance brood was screened towards aeromoniasis as a trait in the rohu breeding programme, resulting in greater improved survival rate during a challenge study which was subsequently validated with molecular tools (Mahapatra et al., 2008). The single nucleotide polymorphisms were found to be informative and have been used to create a linkage map to analyze the QTLs affecting resistance to *A. hydrophila* (Robinson et al., 2014). To address seasonal spawning behaviour, a transcriptome profile generated for twelve-tissues from pre-spawning stage by mRNAseq using IlluminaGA-II-platform (Sahu et al., 2015); whereas, genetically improved rohu has accomplished nearly 17% genetic gain per generation and has been named Jayanti (Gjerde et al., 2019). Recently in India, completed rohu draft genome (PRJNA437789) contains protein coding sequence approximately 32,379 as reference proteome in UniprotKB database (Das et al., 2020). Transcriptome sequencing was analysed in the liver for the diet containing high glucose in Jayanti rohu and revealed differentially expressed genes associated with glucose metabolisms (Rasal et al., 2020). Reproductive seasonality was observed in rohu gonads, liver, brain and pituitary for identifying biological clock genes (BCGs) using Illumina HiSeq 2000 platform (Jaiswal et al., 2021). A comparable Illumina platform was utilised to detect differentially expressed genes and SNPs in the body weight of genetically enhanced rohu (Nandanpawar et al., 2023). After whole genome sequence of rohu, another research group were studied using high-quality de novo genome combination of next-generation sequencing, revealed 946 Mb genome with 2,844 unplaced scaffolds and 25 chromosomes (Arick et al., 2023). In our previous study, mass spectrometric workflow was developed for liver using FASP, S-Trap, and in-solution proteome in rohu (Nissa et al., 2022a).

2.5. Proteomics revolution

The term “proteomics” was first used by Marc Wilkins in 1996 to denote the “PROTein complement of a genOME” (Wilkins et al., 1996). Proteins are particularly significant research targets because they are the effectors of

biological processes and are exposed to regulations that are not found by genomes or transcriptomics studies. Furthermore, although the protein coding gene may be present, it may only be triggered during certain cellular events or external circumstances. Measurement of mRNA transcripts provides a quantitative assessment of the transcription of a given gene, but this quantity is just a rough estimate of the final protein level due to regulation of protein translation and protein degradation. The global analysis of proteins, which are essential functional elements in the cell, are probably the main level of information required to understand how cells communicate; such analysis is referred to as proteomics. Prior to proteomics, the identification of proteins of interest could only be achieved via protein sequencing through Edman degradation. Proteomics is a multidisciplinary science, these include protein separation science, mass spectrometry (MS), and bioinformatics, and these have been described as the strong pillar on which proteomics technologies stand (Tyers and Mann, 2003). Recently improved proteomics techniques provide vast opportunities for better biological understanding of a particular health or disease environment (Cravatt et al., 2007).

2.5.1. Proteomics in Indian aquaculture

In India, proteomics research has found a wide range of applications in human and animal but it has found little progression recently in fisheries science (Mohanty, 2005). So far, in India 76 institutes were actively working in various fields of proteomics and among them, four institute namely, Indian Council of Agriculture Research (ICAR), New Delhi; Kalinga Institute of Industrial Technology (KIIT University), Bhubaneswar; Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad and Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, Bhubaneswar initiated proteomic work in fish (Reddy et al., 2015). The first fish database FISHPROT (http://www.cifri.ernet.in/Fishprot/index.html) in India was developed by the Central Inland Fisheries Research Institute (CIFRI), Barrackpore, especially for biomarker discovery and evaluation. The liver proteome of the murrel *Channa striatus*, the muscle proteome of catla, the plasma proteome of rohu, and the muscle and lens proteome of *Rita rita* are all included in FISHPROT, an online

database, which was developed using Apache, PHP, and MySQL, open source technologies. In the fish cell line first time in India gel based proteomics (2-DE) was used to identify protein signature (Goswami et al., 2015a). In rohu, initially liver proteome was identified using 2-DE (Goswami et al., 2015b). Recently, our team first time in aquaculture peptideAtlas and fish proteome map for 19 tissues of rohu. The developed database would address the most critical challenge of ensuring basic biology, and disease pathways, and designing new therapeutics to achieve sustained production (Nissa et al., 2022b). In our previous study, mass spectrometric workflow was developed for liver using FASP, S-Trap, and in-solution proteome in rohu (Nissa et al., 2022a).

2.5.2. Types of Proteomics

Mainly there are three proteomics i.e. structural, expression and functional. By employing structural proteomics, one can discover the precise location of every protein contained within an organelle or protein complex, as well as the characteristics of every protein-protein interaction. Through structural proteomics, the isolation of complexes nature of protein is easier by purification (Jung et al., 2000). Functional proteomics aims to uncover the biological role of groups or specific proteins, and classes of proteins, on a broad scale across the proteome. To separate particular protein types or protein complexes, protein ligands may be utilized (Graves and Haystead, 2002). The quantitative analysis of protein expression in samples that differ by a given variable is known as differential expression. This technique allows for the comparison of protein expression across the entire proteome across samples, identifying disease-specific markers for focused proteomics (Graves and Haystead, 2002). Among all types, differential proteomics is important in toxicity and disease control in aquaculture.

2.6. Quantitative Proteomics

Quantitative proteomics trace differentially regulated proteins in different sample groups using two categories, such as absolute and relative quantitative proteomics. Quantitative proteomics uses modern proteomics technology, which offers mass spectral data for non-biased protein quantification; in other

words, many proteins were identified in samples. There are different approaches using label and label free for quantitative proteomics available to quantify the protein. It is an extension of expression proteomics as it offers quantitative information on existing proteins within a sample. The information of quantitative protein can also headed for modelling of biochemical networks. In the following context the most developed quantification methods first time used in Indian aquaculture research.

2.6.2. Mass Spectrometry (MS) based proteomics

A mass spectrometer is a tool that uses ion generation, separation, and detection to determine the mass-to-charge ratio of ionised molecules. The instrument offers extremely high sensitivity and specificity. MS based proteomics is a powerful front line of protein-centered technology to identify proteome profile (Sharma et al., 2015, Williams et al., 2016), biomarker discovery (Muntel et al., 2015, Keshishian et al., 2015), post translational regulation investigation (Zhao and Jensen, 2009), protein ligand interaction (Frei et al., 2012), study protein degradation and protein-protein interaction (PPI) (Collins et al., 2013, Rinner et al., 2008). MS-based proteomics can be subdivided broadly in two categories: top-down proteomics (intact protein measurement) and bottom-up proteomics (peptides measured as proxy for proteins). In the context of biomarker studies aiming to identify diagnostic factors, the bottom-up MS acquisition strategy includes discovery and validation analyses. A classic MS consist of an ion source, which produces the ions, a mass analyser, where these ions are parted and a detector, which delivers the mass spectrum (Aebersold and Mann, 2003). In both MALDI and ESI are soft ionisation techniques majorly used for proteomic applications. In MALDI, the sample is mixed with a suitable matrix and co-crystalized wherein sample is ionised using a laser beam in an automatic manner. Desorption predominantly yields singly charged ions from the sample, which are then sorted by their m/z ratio prior to detection (Singhal et al. 2015). In case of ESI, transfer of ions occurs from solution to gas phase with the use of electrical energy and multiple charged ions are separated and detected in mass spectrometer (Ho et al. 2003). The ESI method is particularly useful for the production of ions from macromolecules since it overcomes the tendency of

these molecules to fragment when ionized. In contrast to other atmospheric pressure ionization processes, ESI produces multiply charged ions, thereby extending the mass range of the analyzer to accommodate proteins of varying magnitude and associated polypeptide fragments.

2.6.3. Types of quantitative proteomics

2.6.3.1. Label based quantification

Bottom-up MS has few practical constraints e.g. identical quantities of multiple peptides may not produce equal MS signal, due to variation in ionization efficiencies leading to ion suppression. Numerous quantitative proteomic techniques based on chemical isotope labelling have been developed to address these issues. By comparing the labelled samples with differential mass tags, label-based quantitation enables detection based on a particular change in mass. In order to use label-based approaches, samples from various environments containing stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O) must be analysed quantitatively in a mass spectrometer after being in-vivo or in vitro. The introduction of the isotopic label can be achieved through metabolic labelling, chemical or enzymatic labelling, SILAC (stable isotope labelling by amino acids in cell culture), TMT (tandem mass tag), and iCAT (isotope coded affinity tag). The three primary components of the isobaric mass tags are the reporter group, the mass-balancing group, and the peptide reactive group, which reacts with N-terminal amino groups to label peptides and the amino groups of their lysine residues. Peptides are labelled with an amino-reactive reagent in a TMT following the trypsinization of the samples. The TMT reagent comes in various isobaric forms, each with a unique reporter and balance group for the reagent's stable isotopes ^{13}C and ^{15}N (Bąchor et al., 2019). TMT has the same mass of 229 Da and is available in several ranges, such as TMT 6-plex and TMT 10/11-plex tags. Although the reporter ions in the TMTpro 16-plex reagents range from 126 to 134 Da, they are a continuation of the first 11 reporter ions found in the TMT 11-plex reagents. The total mass of each tag in TMTpro 16-plex reagents is 304 Da (Chen et al., 2021). TMT samples that have been later labelled are merged into multiplexes and subjected to data-dependent

acquisition (DDA) mode LC–MS analysis. When the precursor fragments, the reporter portion of the label is liberated and is visible in the fragment ion spectrum. Reporter ions derived from various TMT reagents are identified as discrete peaks whose relative intensities match the relative molar concentration of the fragmented peptide ion in each of the research samples that make up the TMT multiplex sample. This is because these reporter ions have different m/z values. The most widely used isotope labelling method is iTRAQ, which is based on covalently labelling peptide side chain amines and N-termins. Ross et al. (2004) reported that iTRAQ was developed using internal peptide standards to perform relative and absolute quantitation, and that only a small quantity of protein was used for measurement. iTRAQ enables the simultaneous labelling and quantification of four or eight samples, in contrast to iCAT and SILAC, which compare two or three samples (Choe et al., 2007). The relative abundances of each peptide in the samples under comparison were directly correlated with the respective intensities of reporter ions that were obtained.

2.6.3.2. Label free based proteomics

There are still issues are existing in label based method due to the complexity of a crude proteome. Label free proteomics is an evolving field in MS based quantitative proteomics that utilize only the peptides to conclude quantification. The most interesting feature of label-free quantification has been the effective technique for assessing several protein samples by analysing signal peaks and mass spectra following the separation and identification of each LC-MS / MS sample separately (Zargar et al., 2016). In this method, relative quantification is quantified by comparing either the chromatographic peak area of extracted ions or spectral count values. The intensity range usually calculated from either using precursor scanning of intact peptide or fragment spectra via spectral counting of the intensities of the ion series. In LC-MS, the ion with a specific m/z is captured and observed at a given time with a particular intensity. Based on the ratio of m/z, retention time (elution profile) and signal intensities, the area under the curve (AUC) is determined using automated software.

2.7. Differential protein expression of infected fish

Protein expression profiling entails the identification of proteins expressed in a specific tissue during a specified timeframe and under particular conditions, usually in comparison to expression levels in reference samples (Bradley et al., 2009). Differential expression has been a very powerful tool to identify potential cellular components in biological processes, building bases for molecular analyses of phenotypes and their dynamics at a system level (Rossini et al., 2011). One of the major issues for bacterial infections in fish caused by resistant strains are of grave concern in aquaculture. In this context, it is essential to improve the understanding of infectious mechanisms and the host response to develop new drugs or vaccine with potential activity against these pathogens. Proteomics has emerged as a widely utilized technique for addressing the numerous gaps that persist in our comprehension of bacterial infectious processes in fish (Petriz and Franco, 2014). In zebrafish, infected with virus altered glycolytic related pathways, cytoskeleton, vitellogenin and for *Aeromonas hydrophila*, immune response was dysregulated (Encinas et al., 2010, Medina-Gali et al., 2019, Lü et al., 2014). Crucian carp infected with cyprinid herpesvirus-2 proteins like phagosome pathway and p53 signalling pathways were induced for virus (Liu et al., 2018). Cytoskeletal and cellular signal transduction proteins were modified in zebrafish and turbot after infected with (Zhang et al., 2013, Xiong et al., 2011). Against *A. salmonicida* infection in teleost, the immune system is triggered, and acute phase and immune responses in the liver or spleen dysregulated in the rainbow trout (Long et al., 2015). First defense mechanism of Atlantic cod skin mucus against vibriosis showed differentially expressed proteins of the immune system (Rajan et al., 2013). In olive flounder, proteins like cathepsins, heat-shock proteins, and complement components was dysregulated against *Streptococcus parauberis* (Cha et al., 2012). Differentially expressed proteins in kidney and liver of salmonids was identified against enteric red mouth disease like c-type lectin receptor proteins, antioxidants, lysozyme, cytoskeleton and metalloproteinase (Lin et al., 2018).

A proteomics approach was employed to analyze the liver expression of rainbow trout subjected to two diets with varying proportions of plant ingredients (Martin et al., 2003). In zebrafish recombinant proteins used as protective immunogens against *E. tarda* and revealed presence of innate immunity genes encoding IL-1b, IL-6, IL-8, C3b, and NF-κB. Immune related proteins like complement component (C1r, C3, C5, C7, and C9) and plasma protease C1 inhibitor (C1-INH), major histocompatibility complex class I (MHC class I) and proteasome activator complex subunit 1 (PSME1) were identified in the darkbarbel catfish liver, after infection with *E. ictaluri* (Li et al., 2019).

2.8. Pathway analysis for differentially expressed proteins

Protein-Protein interaction (PPI) network

PPI provide a global print for the understanding of biological processes in aquaculture research. PPI interface is the zone between the two protein chains. If the amino acid contents of the two chains are the same, the interface is referred to as a homomeric interface; otherwise, it is known as a heteromeric interface. Protein interaction network analysis can also be carried out to identify different regulatory/ metabolic pathways of protein expression. Advanced mass spectrometry devices and sophisticated analytical methodologies led to considerable gains in the analysis of PPIs in proteomics (Iacobucci et al., 2021). At present, the PPI network is particularly useful in revealing the regulatory mechanisms of innate immunity and has been extensively used to explain the processes of diseases and pathogens in humans, and plants (Park et al., 2019, Nintemann et al., 2017). As more genomic information becomes available and proteomic data in fish becomes increasingly scarce, it will play a bigger role in providing a functional element to gene expression (a change in gene expression is not necessarily correlated to a change in protein). As protein sequence data grows, completely automated computer programmes that can predict interactions between proteins are becoming critical starting points for system-level function inference. Through use of genomics and proteomics in aquaculture species disease management allows for rapid prediction or identification of the proteins that are involved, but not interactions with the other

proteins, and this significantly hinders an in-depth understanding of the disease pathogenesis and subsequent management. Therefore, the application of the PPI network to aquaculture species could provide new insights into their defense mechanisms toward impending stressors (Waiho et al., 2021).

2.9. Targeted Proteomics for identifying biomarkers

In proteomics, a recently established targeted proteomics approach that uses LC-MS to identify relevant proteins and their corresponding peptides serves as a substitute to immunoassay. It is a sophisticated proteomic method for system biology studies across multiple omics domains. Validation is a critical step in ensuring that a biomarker can be reliably identified in huge data sets, following its detection from comparative proteomics utilising a variety of omics approaches. Using spiking peptide, stable isotope-labeled peptides having amino acid sequences similar to the peptides being measured as internal standards, targeted proteomics measures peptides specific for a target protein. It is generally accomplished via targeted data acquisition schemes were selected reaction monitoring (SRM/MRM) and parallel reaction monitoring PRM) methods. Accurate, repeatable, and specific detection of target proteins is possible in a specific proteomic investigation. SRM is performed using a QQQ mass spectrometer**.** The sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) and parallel reaction monitoring (PRM) are employed in the enhanced targeted technique (Shi et al., 2016). When compared to SRM, PRM offers higher mass accuracy and resolution in the detection of fragment ions because the target ion is chosen using Q-TOF or Q-OT instrument configurations. When using a Q-OT setup for PRM, the first mass analyzer, or quadrupole, separates the predetermined peptide. This peptide is then sent via C-trap to the collision cell, or HCD (High Energy Collision Dissociation Cell), for fragmentation, after which all of the fragment ions are sent to the detector (Shi et al., 2016). PRM varies from SRM in that it selects peptides rather than product ions. In recent years, skyline software was created for mass spectrometric based targeted proteomics. Skyline is open source and freely-available end-to-end supporting proteomics software in targeted proteomic mass spectrometry workflow (MacLean et al., 2010). To help with

the establishment of instrument methods for targeted and DIA investigations, a skyline document is initially utilised for assay development.

In fish and fishery products detection of allergens are important to avoid allergic reaction in human. A targeted technique was used to detect airborne shellfish allergens in processing plants, preventing occupational asthma and allergenicity (Carrera et al., 2020). In fish**,** β-PRVBs, which are considered as allergens and found in high amount in sarcoplasmic fraction of the white muscle (Elsayed and Bennich, 1975, Aukrust et al., 1978). Using MALDI-MS, four isoforms of β-PRVB were identified in rainbow trout. In cod, tuna, tilapia and salmon, fructose bisphosphate aldolase (39.54 kDa) enolase, which end product of gluconeogenesis, glycolysis, and the calvin cycle, is also considered a fish allergen and detected using SDS-PAGE, ELISA, 2-DE, and MALDI-TOF MS (Liu et al., 2012). In bacterial infection**,** targeted approach (SRM and PRM) has produced sensitive quantitative results for clinical diagnosis and antibiotic resistance (Saleh et al., 2019). Targeted proteomics studies have also been initiated in fish reproductive biology a candidate sex-related biomarkers observed for gonadal differentiation in zebrafish and validated using SRM (Groh et al., 2013). To check the adulteration of rainbow trout in Atlantic salmon, SRM approach was used to authenticate processed food product and batch variability (Gu et al., 2020). In fish, biogenic amine was monitored using targeted MRM assay using ultra high performance liquid chromatography-triple quadrupole mass spectrometry The detection of fish parasites anisakids PRM based approach was employed, by monitoring parasite specific peptide markers in fish samples (Carrera et al., 2016). Skin mucus is one of important innate immune system in fish and in yellow croaker, thermal-tolerant protein was identified and validated using PRM (Zhang et al., 2021). PRM analysis was used to validate proteins expressed during metabolic liver diseases (MLD) of largemouth bass (Wu et al., 2022); nonalcoholic fatty liver of tilapia (Yu et al., 2021).

Objectives

With this background the present study was conducted to identify differential protein expression in *Labeo rohita* against *Edwardsiella tarda* infection with the following objectives.

1. **To profile proteome data of selected tissues of *Labeo rohita* infected with *E. tarda***
2. **To analyse differentially expressed proteins of selected tissue in *E. tarda* infected *L. rohita* using label based (TMT) proteomics.**
3. **To analyse the protein-protein interaction of differentially expressed protein of the selected tissue in *E. tarda* infected *L. rohita.***

3. Methodology

3.1. Collection and maintenance of rohu stock

Four 500 l capacity fiberglass reinforced plastic (FRP) tanks were selected, cleaned, disinfected with chlorine (10ppm), and dried. All the tanks were filled with dechlorinated water and were labelled as C_1, C_2, C_3, and C_4. To protect the fish, all the tanks were covered with nylon net on the top of FRP tanks. A total of 200 healthy *Labeo rohita* with an average weight of 100 g were procured from Powarkheda regional centre of ICAR-CIFE, Madhya Pradesh and brought to central wet lab facility ICAR-CIFE, Mumbai, India. The fish (n=200) were brought to the wet lab, disinfected with 5 ppm potassium permanganate for 10 min and stocked in the 500 l capacity FRP tanks at a density of 50/tank containing 400 l water. The fish were acclimatized for about two weeks, and during this period they were fed with commercial feed (GodrejAgrovet-20mm pellet size) at a rate of 2% body weight. Meanwhile, accumulated faeces were removed daily and 50% of the water was exchanged once a week.

Water quality of the tanks was maintained. The water temperature varied from 28 ± 2 ^{0}C and the pH of the water varied from 7.4 to 7.6 during the experiment. To confirm *E. tarda*-free rohu, randomly fishes selected from stock tanks and kidney, liver of ten fishes used for bacterial swab test using in SS-agar plate.

3.2. Bacterial strain and media

Edwardsiella. tarda (ATCC 15947™ culture) stock was obtained from the Aquatic Animal Health Management Laboratory, ICAR-CIFE, Mumbai. *E. tarda* was revived using brain heart infusion (BHI) broth from culture loop and incubated for 18 hr. Before the infection study, bacterial culture was streaked on Salmonella-Shigella Agar (SS agar), selective medium and incubated at 28 ± 2℃. A single black centre colony grown on SS agar plate was inoculated in BHI broth again and incubated at 28 ± 2 ℃ for 22 h. The bacterial culture was immediately stored at -80 ℃ using 20% glycerol stock for the entire experiment. Before challenge dose, one colony of the *E. tarda* were aseptically picked from SS agar, transferred to 10 ml BHI broth separately, and incubated at 28 ± 2°C for 18 h. Later culture was centrifuged at 6000 rpm at 4 °C for 10 min to collect the cells. The pellets thus obtained were washed three times with 1X phosphate

buffered saline (PBS) and suspended in 10 ml saline. The numbers of bacterial cells in the saline suspensions were determined by spread plating on nutrient agar plates.

3.3 Determination of lethal dose (LD_{50})

3.3.1 LD_{50} calculation of *Edwardsiella tarda* in rohu

The glycerol stocked *E. tarda* was thawed first, then inoculated in BHI broth for 18 hr and cultured at 28 ± 2 ℃. An appropriate amount of bacterial culture was centrifuged at 6,000 rpm for 10 min, and the pellet was washed thrice with 1X PBS and re-suspended in PBS. The optical density of bacteria was calculated using a spectrometer at 600 nm (OD_{600}) and adjusted to 0.6. The LD_{50} dose used for the challenge was determined by intraperitoneal injection into rohu at 28 °C. A total of 120 apparently healthy rohu fish (100 ± 20 grams) were selected and kept for acclimatisation in the crates for one week and divided into two groups. The first group of 20 healthy fish was given 0.1 ml of sterile PBS (control group). In the second group, fish were randomly divided into five groups. Each group contained 20 fish and was injected ten-fold dilution ranging from 10^9 to 10^5 cfu/ml intraperitoneally. The death rate was monitored for up to 7 days, and the LD_{50} was determined using the Reed-Muench method (Reed and Muench, 1938).

3.4.1. Pathogenicity test

A total of 36 healthy *Labeo rohita* with an average weight of 100 ± 20 g were selected for expression study. The fish was confirmed as non-infected rohu with negative results for *E. tarda* by gill, kidney and spleen swab in a selective medium. The fish were maintained in plastic tanks (crates). Prior to the challenging experiment, all the fish were adapted to the conditions of the tanks for two weeks. The fish were randomly divided into 2 groups, including the control, and *E. tarda* infected groups (n=18 for each group). Each group was reared in three tanks (6 fishes/tank). The fish from the infected groups were intraperitoneally injected with 0.1 ml of *E. tarda* at concentrations of 1.6×10^7 cfu/ml (diluted by Phosphate buffered saline medium), respectively. The fish from the control group were injected with 0.1 ml of sterile PBS medium. The fish was euthanized using clove oil and the kidney, liver, gut tissues from all the fishes were collected at 48h post-injection and stored at -80°C after dry ice

freeze. For reconfirming bacteria selective media used.

3.5 Protein exaction protocol for diseased rohu

3.5.1 Tissue lysis and protein extraction

Tissue lysates were prepared using SDS containing lysis buffer (5% SDS, 100mM Tris/HCl pH 8.5 (adjusted with phosphoric acid). Briefly, 40-50 mg tissue was weighed and washed with 1X phosphate buffered saline (PBS) solution (2-3 times to remove any blood). After washing the tissue, 250 µl of buffer 1 along with 5 µl of Protease inhibitor cocktail (Sigma- 50x stock) was added to the tissue and incubated on ice for 30 minutes. Sonication was performed for 2 minutes with an amplitude of 40% with 5 sec pulse on and 5 sec off. (This step was repeated thrice for gut tissue and amplitude was increased to 55%). Centrifugation was done at 8000 g for 15 min to remove the debris and clear supernatant was collected.

3.5.2 Protein quantification and digestion

Tissue lysates were processed for quantification of proteins using BCA kit (Thermo, ref No 23227) method using Bovine serum albumin as standard protein. Briefly, standard curve was prepared for the known protein concentration range of (5-250 µg/ml BSA). The working reagent (WR) was prepared by adding 50 parts of BCA Reagent A and 1 part of BCA Reagent B (50:1, Reagent A:B). Use 5µl of standards, blank (distilled water) and unknown samples into the microwell plate and add 200µl of WR to the microplate, then incubate for 30 minutes at 37°C. Measure the absorbance at or near 562 nm on a plate reader. For identifying concentration of unknown samples, subtract the average 562 nm absorbance measurement of the blank standard replicates from the other individual standard and unknown sample replicates. Prepare a standard curve by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

3.5.3. Filter-aided sample preparation (FASP) based digestion of proteins

After quantification 30 µg protein was taken for digestion using FASP based digestion method. Briefly, 30 µl of protein extract was added to the filter unit and reduced with TCEP solution in 1x lysis buffer (20mM) to reduce the protein by incubating it at 37 ℃ for 1 h. Later, 30µl of a protein extract was mixed well

(30 ug protein here) with 200 µl of UA in the filter unit (30 kDa) and centrifuge at 14,000 x g for 15 min. Again, mix up step of UA was repeated. In the collection tube, discard the flow-through and add100 µl IAA solution. Mix at 600 rpm in a thermo-mixer for 1 min and incubated in the dark without mixing for 20 min. Centrifuge the filter units at 14,000 x g for 10 min. Add 100 µl of UA to the filter unit and centrifuge at 14,000 x g for 15 min. Repeat this step twice. Add 100 µl of ABC to the filter unit and centrifuge at 14,000 x g for 10 min. Repeat this step twice. For trypsination step, added 40 µl ABC with trypsin (enzyme to protein ratio 1:30) and mixed at 600 rpm in thermo-mixer for 1 min. Incubated the units for 4-18 h in a wet chamber at 37 °C. The incubated filter units were transferred to new collection tubes and centrifuge the filter units at 14,000 x g for 10 min. Add 40 µl ABC and centrifuge the filter units at 14,000 x g for further 10 min. Dry the eluted peptides. Before mass spectrometry, samples were cleaned using C18 stage tips.

3.6. Tandem mass tag (TMT) analysis for rohu gut

TMT six-plex isobaric tags (TMTsixplex™ Isobaric Label Reagent Set, 1 × 0.8 mg, Thermo Fisher Scientific, Catalog number: 9006) were used for labelling of the digested peptides of rohu gut (CG_1-126, CG_2-127 and CG_3-128; ETG1-129, ETG_2-130 and ETG_3-131) as per the manufacturer's instructions. In brief, allow the vial of TMT 6-plex reagent to reach room temperature RT for 5 min, then spin the solution in the vial (2 min) to bring the solution to the bottom. Added 45 µl acetonitrile (ACN) to TMT reagent, vortexed before adding to peptides. TMT reagents (40 µl) were added to the corresponding aliquot of digested gut peptides following the labelling strategy; 50 µg of each digested peptide sample was labelled at ~1:13 ratio (digested peptides: TMT reagents) for performing efficient labelling. The solution was mixed well and incubated at room temperature (RT) for 1 h. The reactions were quenched by adding 2 µl of 5% hydroxylamine and were incubated for 30 min at RT. Combine the contents of all TMT reagent -labeled sample tubes into one tube. All the respective samples were dried completely in a vacuum centrifuge. Samples were then fractionated into fractions using high-pH reverse-phase chromatography following the manufacturer's instructions (Pierce™ High pH Reversed-Phase Peptide Fractionation Kit, Thermo Fisher Scientific, Catalog number: 84868).

3.7. Liquid chromatography tandem mass spectrometry in data dependent acquisition mode

For all the samples (Control and ET group), peptides were quantified using the Scopes method (Scopes, 1974). One µg of peptide was injected into the mass spectrometer for liquid chromatography-tandem mass spectrometric analysis (LC-MS/MS). All samples were run through a 120-minute gradient on an Easy-nLC 1200 system connected with an Q Exactive Orbitrap mass spectrometer. At a flow rate of 5 µl/min, the peptide sample was loaded onto the trap column (100 µm x 2 cm, nanoViper C18, 5 µm, 100A; Thermo Fisher Scientific). Peptides were separated on an analytical column (3 µm particle, and 100 Å pore size, 75 µm × 50 cm; Thermo Fisher Scientific) at a flow rate of 300 nl/min using solvent A (0.1% formic acid (FA) in water) and solvent B (80% acetonitrile with 0.1% FA). The Orbitrap mass analyzer was used to perform mass spectrometric acquisition in data-dependent acquisition (DDA) mode over the entire scan range of 350-1700 m/z and with a mass resolution of 70,000. The mass isolation window was set at 2.0 m/z with a dynamic exclusion time of 40 seconds. For fragmentation, all MS/MS spectra were obtained using the HCD method (High Energy Collision Dissociation) of 30%. The AGC target for MS1 and MS2 was set at 3e6 and 5e4 respectively. For positive internal calibration, a lock mass of 445.12003 m/z was used.

The liquid chromatography gradient used for label-free quantitation (LFQ) experiment is shown below. It summarizes the time duration for solvent B at different intervals of time with a flow rate of 300 nl/min.

Time [mm:ss]	Duration [mm:ss]	Flow [nl/min]	Mixture [%B]
00:00	00:00	300.00	05.00
05:00	05:00	300.00	05.00
80:00	75:00	300.00	30.00
110:00	30:00	300.00	60.00
115:00	05:00	300.00	90.00
120:00	05:00	300.00	90.00

In case of TMT, fractions of peptides were run through a 90-minute gradient on

an Easy-nLC 1200 system connected with a Q Exactive Orbitrap mass spectrometer. LC-MS/MS LFQ steps were followed with few modification in the MS1 level, a scan range of 375-1700 m/z with a mass resolution of 60,000 was employed. Maximum injection time was set to 50 ms and AGC target was 4e5. The mass tolerance was set as 10 ppm and dynamic exclusion duration was 40 s. For fragmentation (MS2), all MS/MS spectra were obtained using the HCD method (High Energy Collision Dissociation) of 35%. Isolation window was set as 1.2. Orbitrap resolution was set to 30,000 and maximum injection time was set to 54 ms. Data type was chosen as centroid.

3.8. Protein identification using MaxQuant software

The raw mass spectrometry data was analysed using MaxQuant (v1.6.6.0) software against Uniprot protein database for *Labeo rohita* (downloaded on 24.04.2021) using the in-built search engine, Andromeda. All eight raw files were analyzed together using Label-Free-Quantification (LFQ) parameters. Parameters include label type as standard with a multiplicity of 1 and match between runs was checked. Under the instrument, Orbitrap fusion mode was selected and trypsin as protease was opted. A maximum of 2 missed cleavages were allowed. Under fixed modification, carbamidomethylation at Cysteine (+57.021464 Da) was selected and for variable modification, oxidation at Methionine (+15.994915 Da) was selected. Both at protein and peptide level, false discovery level of 1% was set. For decoy mode option, reverse was selected and proteins identified with unique peptide only. For quantitative analysis, LFQ intensities obtained for each sample were selected.

3.8.1. Proteome discoverer for TMT analysis

Raw instrument files were processed using Proteome Discoverer (PD) version 2.2 (Thermo Fisher Scientific). MS2 spectra were searched using the Sequest HT and Mascot (v2.6.0) search engine against *Labeo rohita* fasta from Uniprot database. All searches were configured with dynamic modifications for the TMT reagents (+229.163 Da) on lysine and N-termini of the peptide and oxidation of methionine residues (+15.9949 Da) and static modification as carbamidomethyl (+57.021 Da) on cysteine, monoisotopic masses, and trypsin cleavage (max 2 missed cleavages). The peptide precursor mass tolerance was 10 ppm, and MS/MS tolerance was 0.05 Da. The false discovery rate (FDR) for

proteins, peptides, and peptide spectral matches (PSMs) peptides were kept 1%.

3.9.1. Statistical analysis using Metaboanalyst

Statistical analysis was performed for liver tissue samples taking MaxQuant analyzed files (Pang et al., 2021). The proteomic data of the remaining seven samples were considered for further analysis. Missing values of the features having abundance values in more than 75% of each group were imputed by the KNN algorithm and considered for differential protein expression analysis. Log transformed data without any normalisation was taken for statistical analysis (i.e., t-test and fold change). Two sample t-test (Welch t-test) was performed to identify the significant differentially expressed proteins where a p-value of 0.05 was set as the maximum threshold value. Among all t-test passed proteins, proteins with fold change values 1.5 were considered significantly differentially expressed proteins (DEPs).

3.10. Pathway and protein-protein interaction enrichment analysis

Selected dysregulated proteins obtained from LFQ data were taken forward for functional annotation and biological pathway analysis. The gene ontology classification of dysregulated proteins was performed in the eggNOG (Version 6.0) (Huerta-Cepas et al., 2019). Later, the entire identified abundant proteome with disease and control group gene list was analysed using Metascape (Tripathi et al., 2015). In addition, the network visualization and the protein-protein interaction analysis for selected pathways was done in STRING (version 11.0) (Szklarczyk et al., 2021).

3.11. Validation of differentially expressed proteins using targeted proteins

A set of differentially expressed proteins of liver and kidney tissue was selected from the discovery data for validation using the Multiple Reaction Monitoring (MRM) approach of targeted proteomics. Transition list was prepared using Skyline software (MacLean et al., 2010). In brief, only unique peptides with 0 missed cleavage were considered with +2 precursor charge and +1, +2 product ions. Initial optimization runs were done in pooled peptide samples. After refinement, a final transition list of liver was obtained having 386 transitions corresponding to 13 proteins (9 upregulated and 4 downregulated) and 51 peptides. In kidney, 12 proteins with a total of 434 transitions were selected.

The raw files were further analysed in Skyline and Data was acquired using TSQ Altis Triple quadrupole mass spectrometer (ThermoFisher Scientific). It was coupled with HPLC-Dionex Ultimate 3000 system (ThermoFisher Scientific). A Hypersil Gold C18 column (1.9 µm, 100 x 2.1 mm, ThermoFisher Scientific, USA) was used for peptide separation and a flow rate of 450 µl/min was employed. Buffer system consisted of solvent A Milli Q water with 0.1% formic acid and solvent B as Acetonitrile with 0.1% formic acid. Individual samples (6 each group) were run against the final transition list. A pooled peptide sample was also run at regular intervals to monitor the consistency of runs over the time. Further peptide analysis, refinement and group comparison was done using MSstats inbuilt tool in Skyline where a fold change analysis cut off of 1.5 with p-value ≤ 0.05 was considered as significant.

4. Results

4.1. Lethal dose confirmation of *Edwardsiella tarda* for challenge study

Table 4. Mortality of *Labeo rohita* during the LD_{50} of *Edwardsiella tarda*

No. of bacteria	Mortality ratio	Died	Survived	D	S	M	%
10^8	16/20	16	4	34	4	34/38	84.2
10^7	11/20	11	9	18	13	18/31	58.1
10^6	7/20	7	13	7	26	7/33	21.2
10^5	0/20	0	20	0	46	0/46	0.0

(Where D-Dead ratio; S-Survival ratio and M-Mortality rate)

$$\text{Proportionate Distance} = \frac{\text{Mortality above 50\% - 50\%}}{\text{Mortality above 50 \% - Mortality below 50 \%}}$$

$$= \frac{58.1-50}{58.1-21.8} = 0.219$$

LD_{50} = Dilution 50 % + Proportionate distance

= 7 + 0.219 =7.219

Antilog 7.367 = 1.6×10^7

LD_{50} = **1.6×10^7**

Mortality was observed within 48 h after intraperitoneal injection and in the tenfold dilution, 100% of mortalities occurred in the first dilution (10^9). However, in 10^8 dose, mortalities were 85% observed and reduced in other doses. In the current experiment, mortality of 10^7 showed > 50% and the overall estimated lethal dose (LD_{50}) for *L. rohita* against *E. tarda* was 1.6×10^7 CFU/ml.

4.2. Pathogenicity of *Edwardsiella tarda* in *Labeo rohita*

Infected rohu exhibited clinical signs of hemorrhages at the injected site and rectal hernia in a few fish with red anal opening after 48 h post-injection. Few fish showed swollen abdomen with haemorrhages or discolouration on the surface of the fish as a major sign.

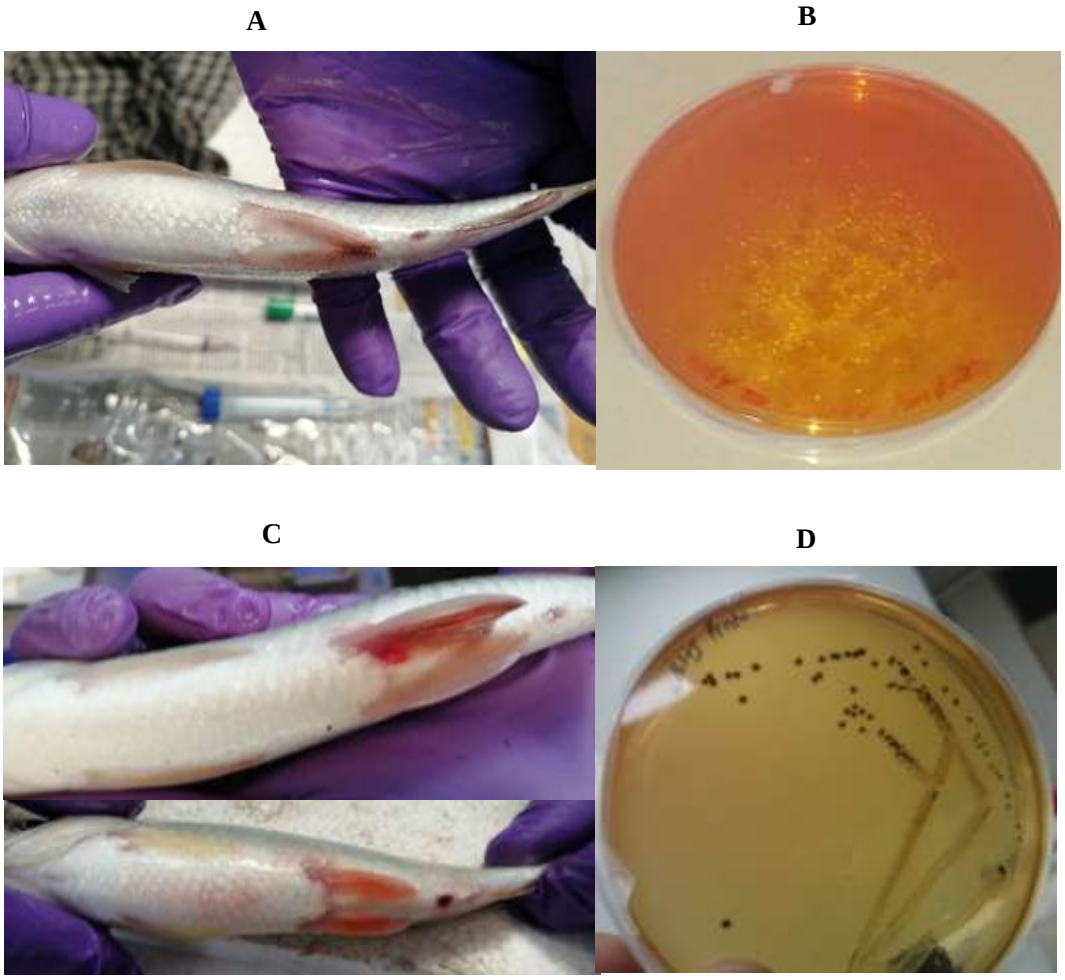


Figure 1. Normal and infected rohu with *E. tarda* showing clinical signs after 48 h (A) Normal healthy fish injected with Phosphate buffered saline (PBS) without clinical signs; (B) PBS injected fish swab showed without black colony in Salmonella shigella (SS) agar plate; (C) Infected fish showed petechial hemorrhage in fin and rectal hernia. (D) Infected fish swab showed black colony in SS agar plate for the presence of *E. tarda*.

4.3. Histopathology of rohu infected with *Edwardsiella tarda*

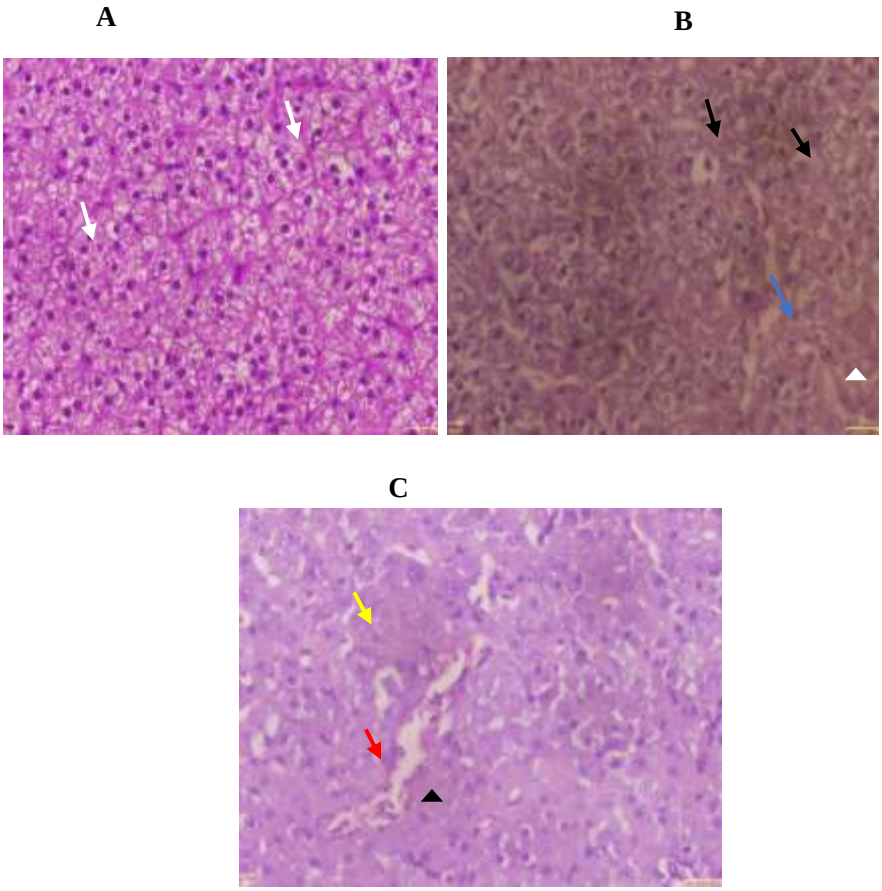


Figure 2. Histopathology of *E. tarda* infected rohu liver; **A**. Normal organization of liver cells from control rohu with kupffer cell (white arrow). **B.** Infected rohu liver showed vacuole formation (black arrow), enlargement of hepatocyte or hypertrophy (white arrow head) and starting stage of infiltration of sinusoids with leukocytes (blue arrow). **C.** Histological changes in the infected liver showed erythrocyte infiltration into blood sinusoids and melanomacrophage centre (aggregation (red arrow), surrounded by kupffer cell (black arrow head) and less nuclei hepatocyte (yellow arrow), Bar = 10 µm.

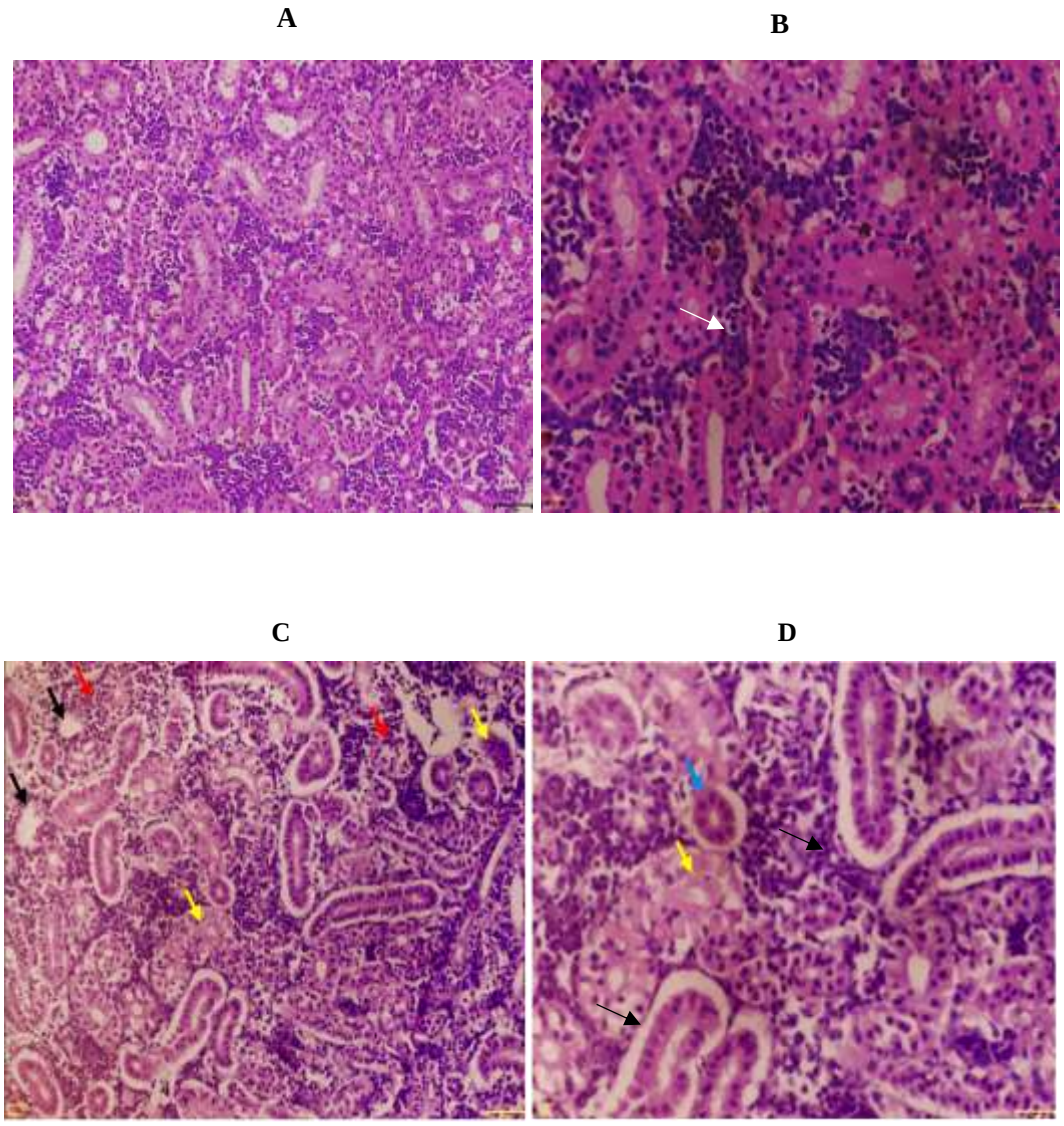


Figure 3. Histopathology of *E. tarda* infected rohu kidney; **A & B.** Photomicrograph of Control/Healthy rohu kidney showed well-arranged lumen of nephric tubules (white arrow); **C & D.** kidney 48 h post infection showing vacuolization (black arrow), and occlusion of tubular lumen cloudy (yellow arrow) swelling degeneration (blue arrows), and necrosis of tubular epithelium (red arrows). Bar = 10 µm.

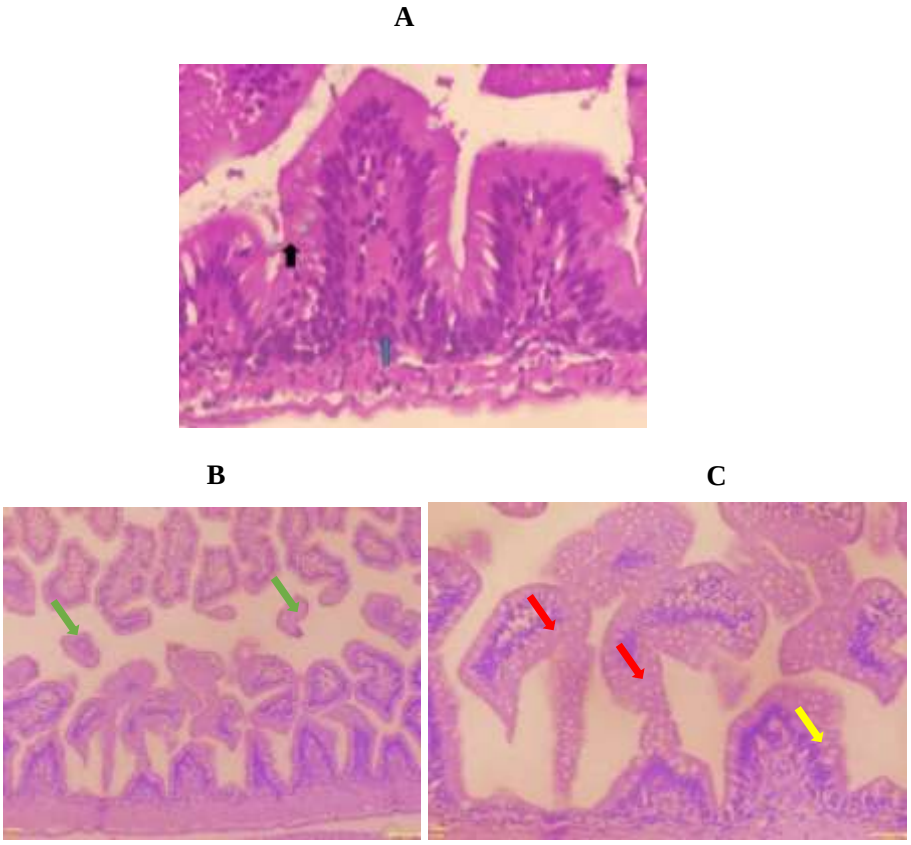


Figure 4. *E. tarda* infected rohu gut; A. control gut showed well-arranged goblet cell (black arrow) without degeneration (blue). In the gut, degeneration submucosa epithelium (green arrows) the villus epithelium goblet cell hyperplasia (red arrow) and hypertrophy (yellow arrow). Bar = 10 µm.

4.4. Tissue-specific proteome profile of rohu

The output file from the MaxQuant and Proteome Discoverer analysis was taken for further analysis. Proteins between two groups having ≥ 2 unique peptides were considered for the analysis and unique proteins obtained from each group were separated for both tissues. In the liver, a total of 1,320 proteins were found across replicates, whereas in the kidney 1,276 proteins were identified. A total of 651 proteins were shared across the group (Fig. 5A)

After LFQ analysis in MaxQuant, the data were trimmed to obtain highly significant proteins. In LFQ data of the liver average of 1,183 proteins obtained across control and infected groups (Fig. 5B (i.)). Further unique proteins among the groups were recorded for the infected and control liver group i.e., enriched in the control group contains 29 proteins and concurrently 108 proteins were enriched in *E. tarda* group (Table 5). While in the kidney, a total of 1,144

overlapped proteins were expressed in both the control and infected groups (Fig. 5B (ii.)). The unique proteins were enriched in the control group 33 and concurrently 99 proteins were enriched in the *E. tarda* infected group (Table 6). In label-based TMT analysis, a total of 1,630 proteins were selected for metaboanalysis (Table.5).

Table 5. Tissue specific proteome profile having ≥ 2 unique peptides used for Metaboanalyst analysis

Tissue	Tissue wise proteins	Common protein across tissues	Unique proteins	Metabo - analyst used proteins	DEPs	Up regulated	Down regulated
Liver	1320	652	343	1183	276	264	12
Kidney	1276		259	1144	96	53	43
Gut	1630		575	1630	29	15	14

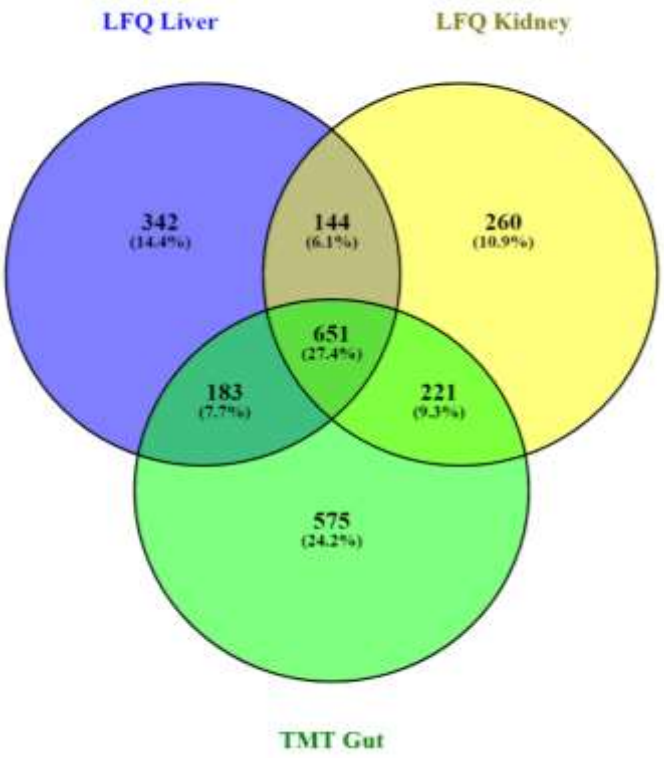
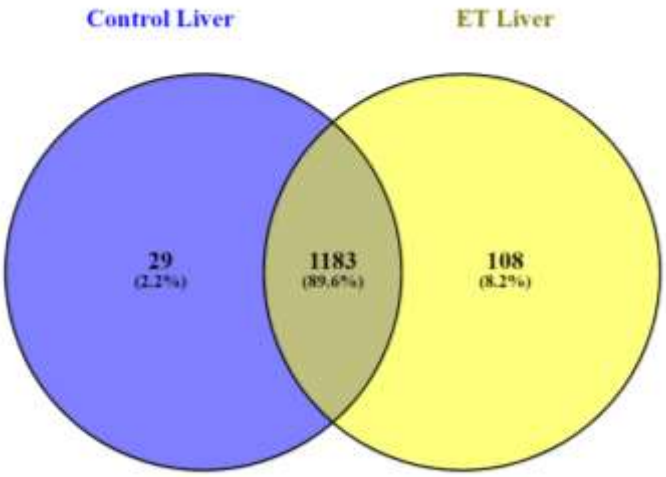


Figure 5A. Overall identified proteome across three tissues of rohu; Proteins represented in liver (blue circle), kidney (yellow circle), and gut (green circle) conditions. Overlapped/share proteins across three tissues are highlighted.

i.



ii.

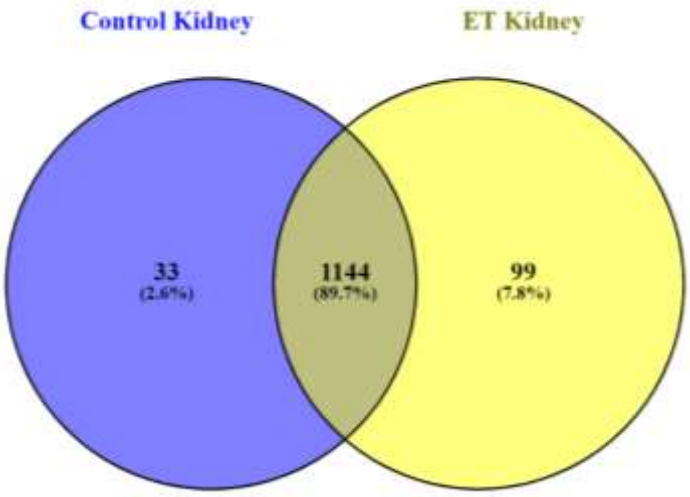
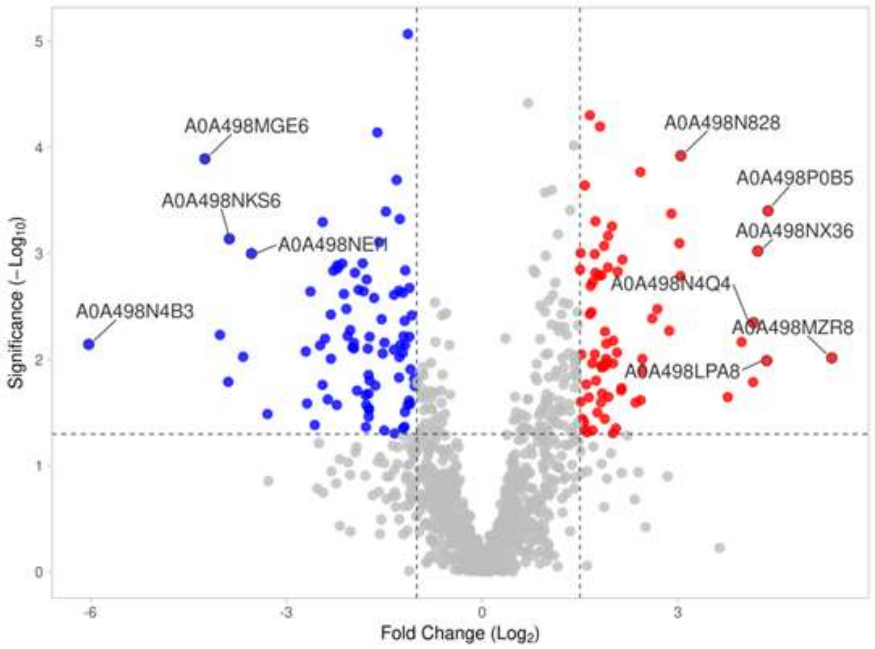


Figure 5B. Venn diagram showing overall quantified proteins; Proteins represented in unique infected (yellow circle) and control (blue circle) conditions, also overlapped/share proteins along with proteins quantified only in one group; (i) liver and (ii) kidney

4.5.1. Protein expression of rohu liver, kidney and gut against *Edwardsiella tarda*

For the quantification approach, statistical analysis was performed in metaboanlyst (5.0) for all the identified proteomes in the liver kidney and gut. The number of differentially expressed proteins in the liver was 276 of which 257 were showing increased abundance, while 20 proteins showed a decreased abundance (Fig. 6(i)). Further unique proteins among the groups were recorded for the infected and control liver group i.e., enriched in the control group contains 37 proteins and concurrently 136 proteins were enriched in *E. tarda* group (Fig. 8(i)) In the case of the kidney, 53 proteins were upregulated, whereas 43 proteins showed decreased abundance, representing a total of 96 differentially expressed proteins during *E. tarda* infection (Fig. 6(ii)). Apart from DEPs, unique proteins between each group of kidneys, i.e. 33 proteins were enriched in the control group and concurrently 99 proteins were enriched in the *E. tarda* infected group (Fig. 8(ii)). A total of 29 proteins were differentially expressed in the gut. Among these DEPs, 16 proteins were upregulated and 15 were downregulated for *E. tarda* infection (Fig. 6(iii) and Fig. 7 (iii)). Representative plots for comparative protein abundance of some of the differentially expressed proteins of liver are shown (Fig. 7(i)) including Intelectin 2 (intl2), Apolipo A-I-like protein (Apoa1) and Metalloreductase STEAP4 (steap4) showing increased abundance. Proteins like Prosaposin-like isoform X1 (psap), Glutathione transferase (gst), Ricin B-type lectin domain-containing protein (OSTbeta) showed a decreased abundance in the ET group (Fig. 7 (i)). In kidney, Metalloendopeptidase (bmp1a), Arachidonate 5-lipoxygenase-like protein (alox5), Transmembrane emp24 domain-containing 10 (tmed10) were downregulated whereas Complement factor B-like protein (cfbl), Metalloreductase STEAP4 (steap4), Mannose-binding C-like protein (mbl2) were highly abundant after 48h of *E. tarda* infection (Fig.7 (ii)).

i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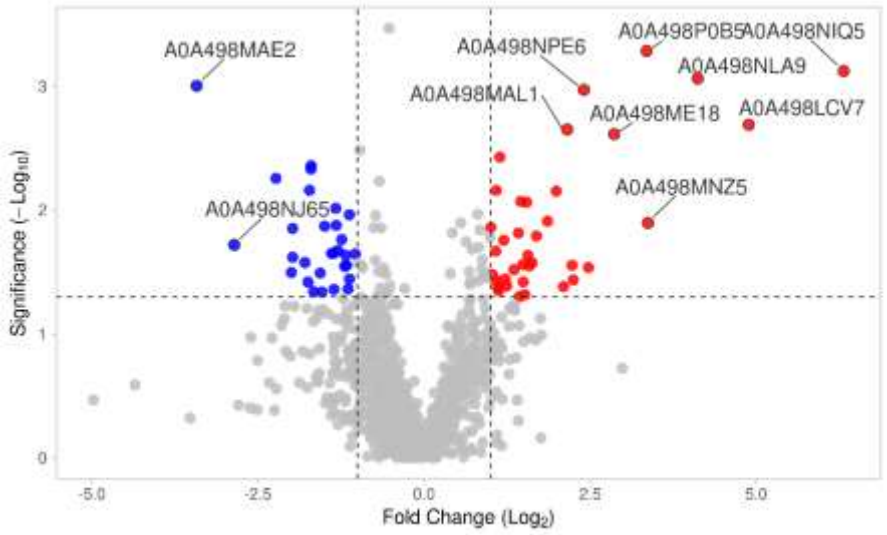
Significance (−Log10)
Fold Change (Log2)

ii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Significance (−Log10)
Fold Change (Log2)

iii

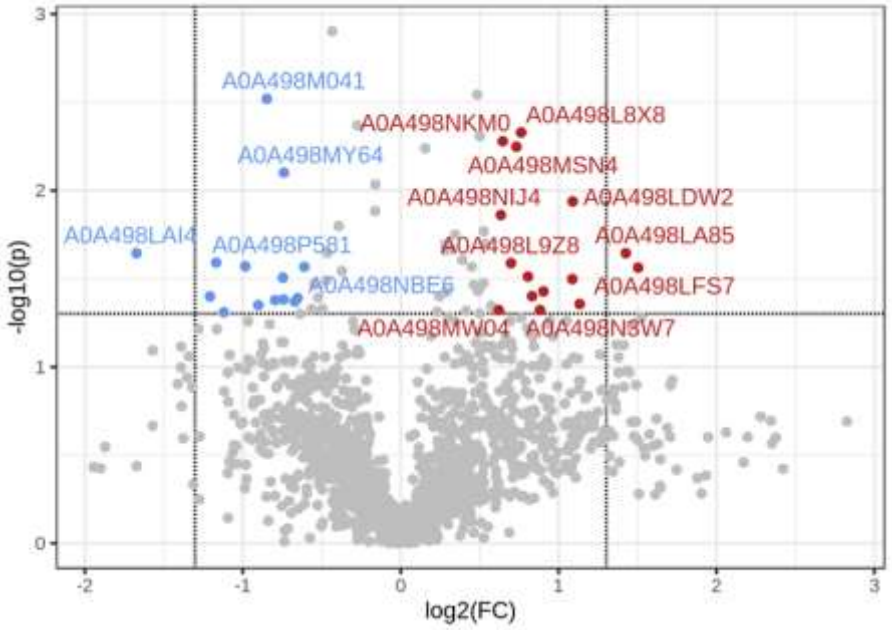
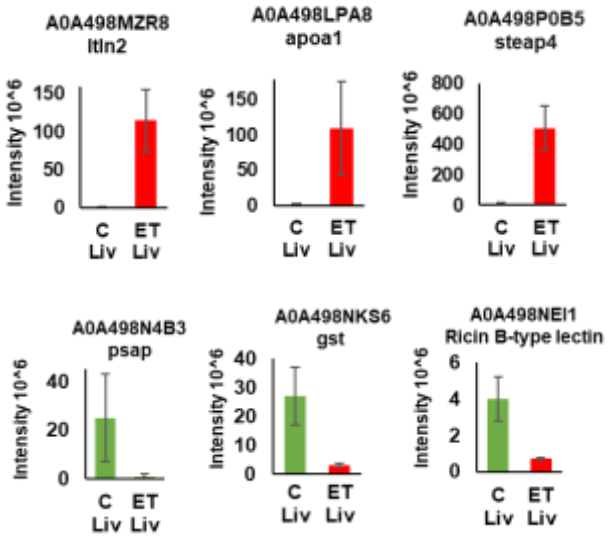


Figure 6. Volcano plot depicting highly significant protein candidates (Fold Change of 1.5, p<0.05), in (i) liver, (ii) kidney and (iii) gut, where blue and red colours represent downregulated and upregulated proteins respectively.

i



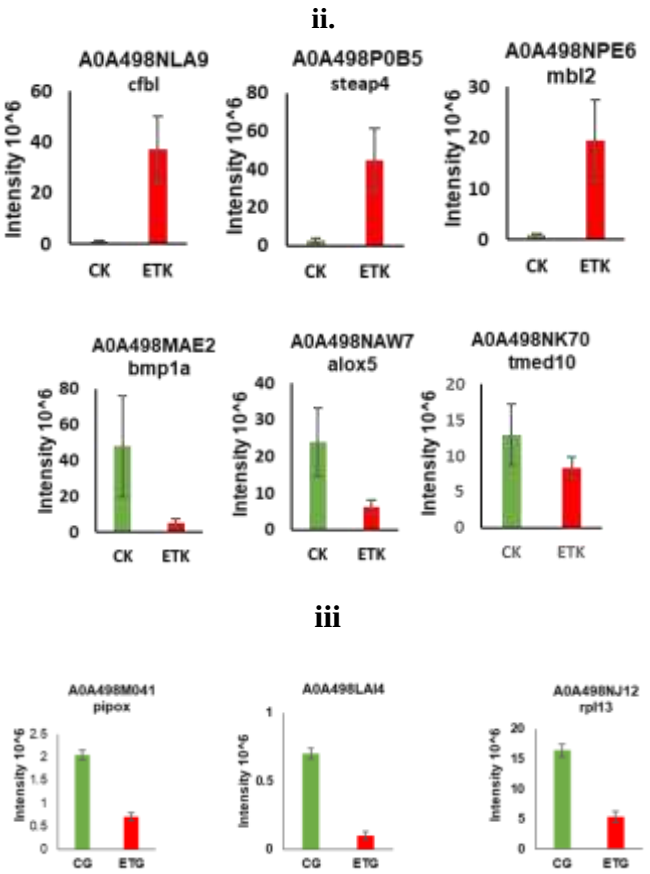


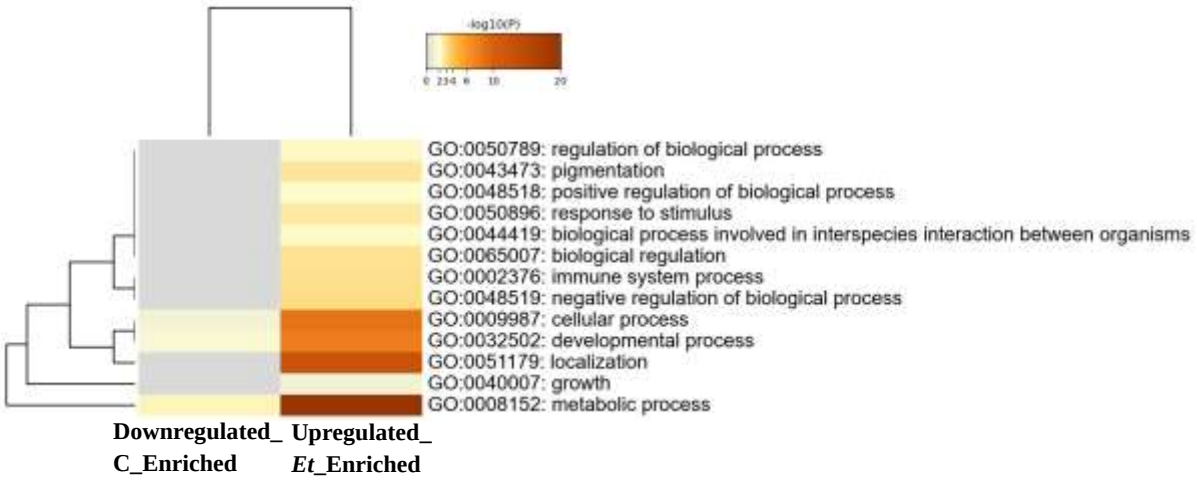
Figure 7. Barplots representing altered abundances of functionally important proteins across control and *E. tarda* infected group of liver (i), kidney (ii) and gut (iii).

4.6. Functional analysis of dysregulated and unique proteins identified in the liver and kidney

4.6.1. Gene ontology of the differentially expressed proteins

For functional analysis (gene ontology), both the differentially expressed proteins (DEPs) and proteins quantified in only one group (enriched) were considered with metascape. The one-group protein expression was considered as a unique protein for the *E. tarda* infection. In the case of the kidney, a gene list was acquired from gene ontology (209 genes) for DEPs. A total of 188 genes were mapped in the metascape and were grouped based on their pattern of gene expression in heatmap and functions for DEPs and enriched DEPs are shown in Fig. 8 (i) liver and Fig. 8 (ii) kidney.

i.



ii.

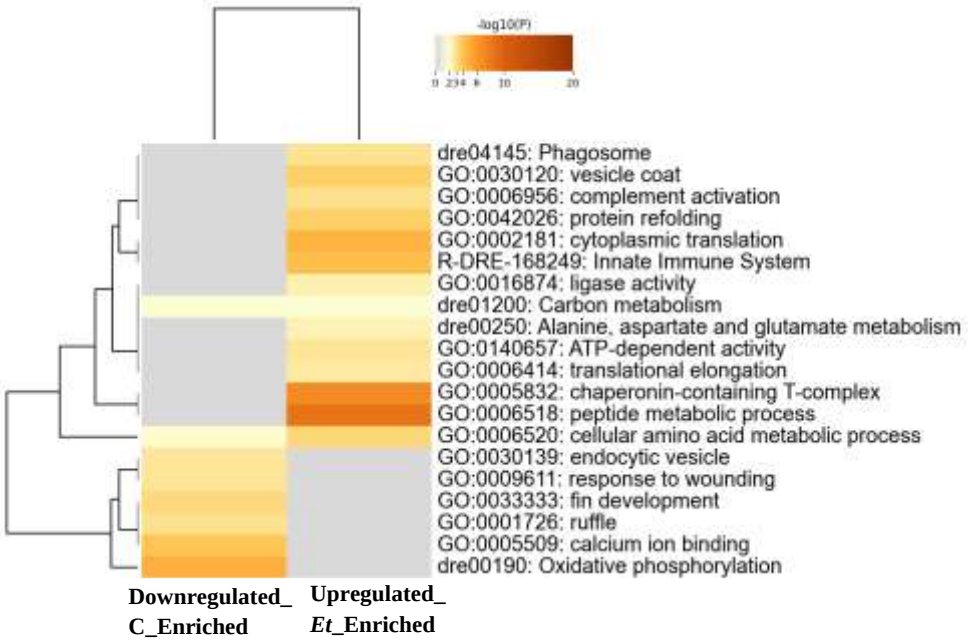
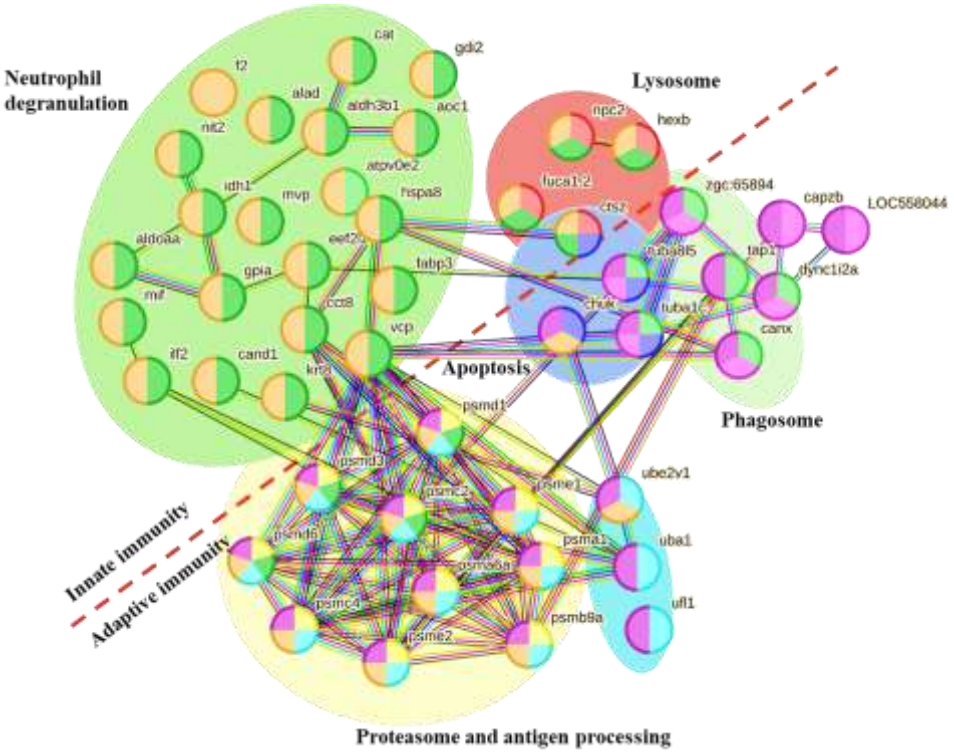


Figure 8. Heatmap of enriched protein in terms of gene ontology for Control (C) and *E. tarda* group (ET); Heatmap for statistically enriched categories of Downregulated and control enriched vs Upregulated protein and *E. tarda* enriched proteins in liver (i) and kidney (ii).

4.7.1. Protein-protein interaction (PPI) of identified immune proteome across tissues

In the isolated proteins across three tissues, a total of 651 proteins were common (Fig. 5A) and in SRING analysis 47 immune proteins were identified Fig. 8 (i.). Proteins involved in the innate immunity were belongs to pathways of neutrophil degranulation and lysosome. While in adaptive immunity, proteins involved in phagosome, apoptosis, proteasome and antigen processing. For unique proteins, in gut 575 proteins used for STRING analysis and 41 immune proteins were identified Fig. 8 (ii.). In gut proteins belongs to signaling of interleukin, cytokine signaling, phagosome, salmonella infection, and lysosome. In liver and kidney few immune proteins identified Fig. 8 (ii.) and involved in drug metabolism, lysosome, sphingolipids, insulin signaling, toll like receptor signaling, PPAR signaling and apoptosis.

i.



ii.

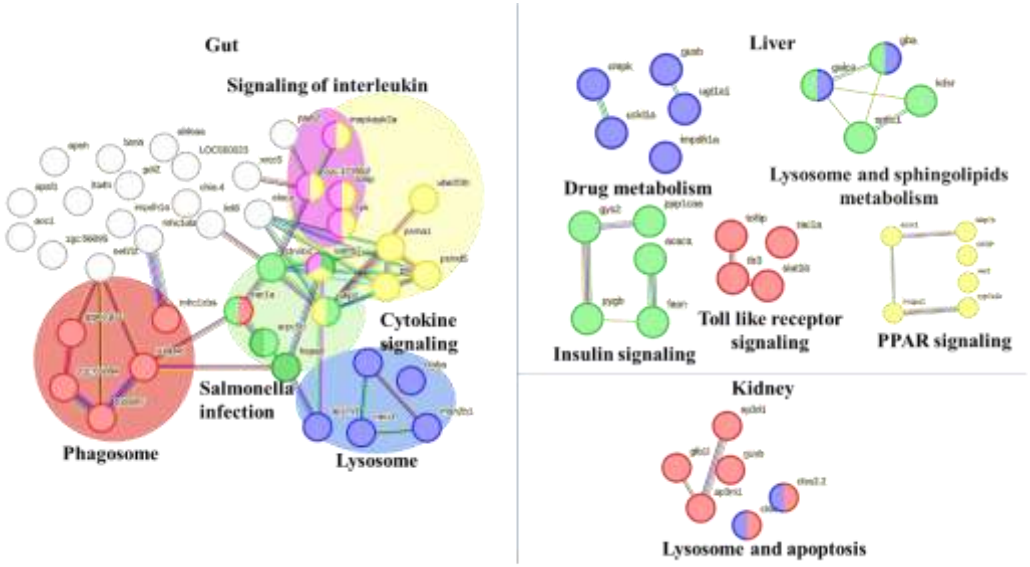
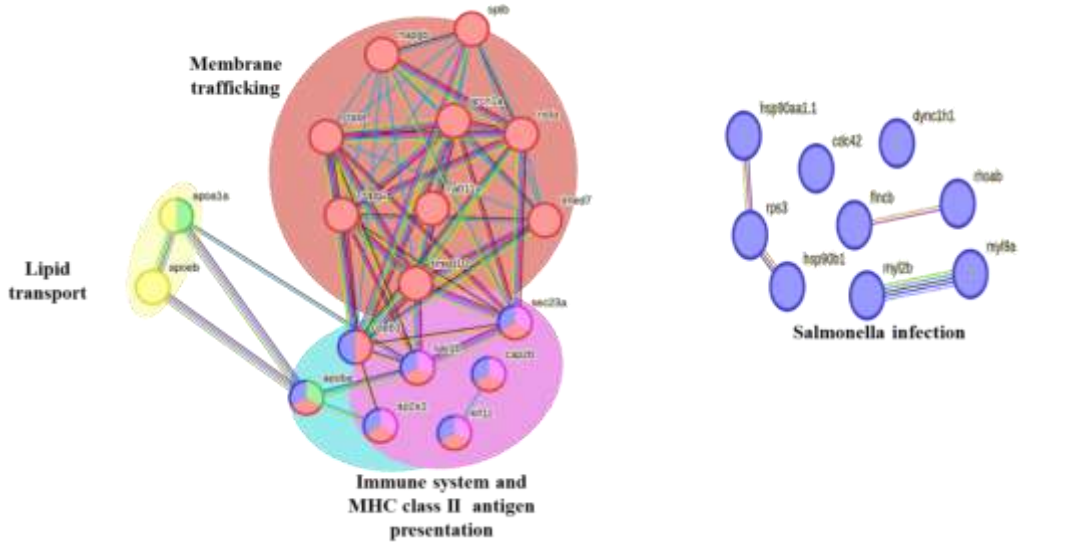


Figure 8. Landscape of immune pathways and interaction networks: Protein-Protein interaction of shared proteins across three tissues involved in innate and adaptive immunity (i.). Tissue wise (gut, liver and kidney) unique immunity proteins were highlighted (ii.)

4.7.2. PPI of differentially expressed immune proteins across tissues

In the *E. tarda* infected rohu clusters of protein association network was obtained in upregulated liver and kidney using STRING analysis. In liver, 252 genes were used for STRING analysis and upregulated protein clusters were involved in membrane trafficking, lipid transport, immune system and MHC class II antigen presentation and salmonella infection Fig. 9A. While in kidney, a total of 43 upregulated proteins showed various pathways in STRING. In the upregulated proteins of kidney, majorly involved in innate and adaptive immunity and other proteins involved in c-type lectin receptors, phagosome, ROS and RNS production in phagocyte, cytokine signaling and interleukin (Fig. 9B). The gut proteins did not show connection to each other in protein-protein interaction analysis.

A



B

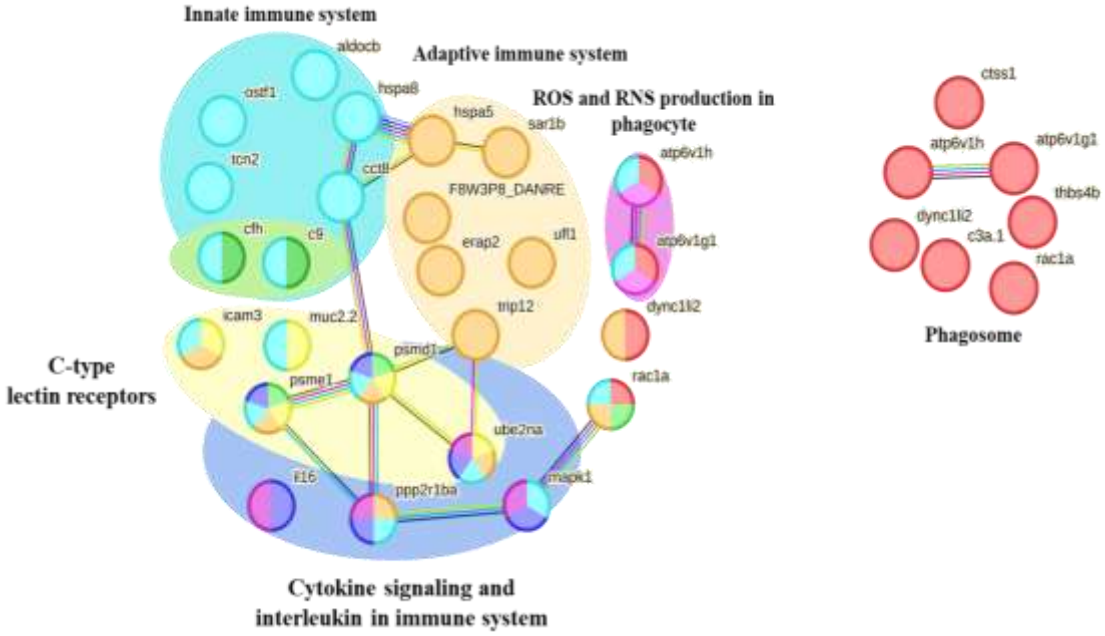


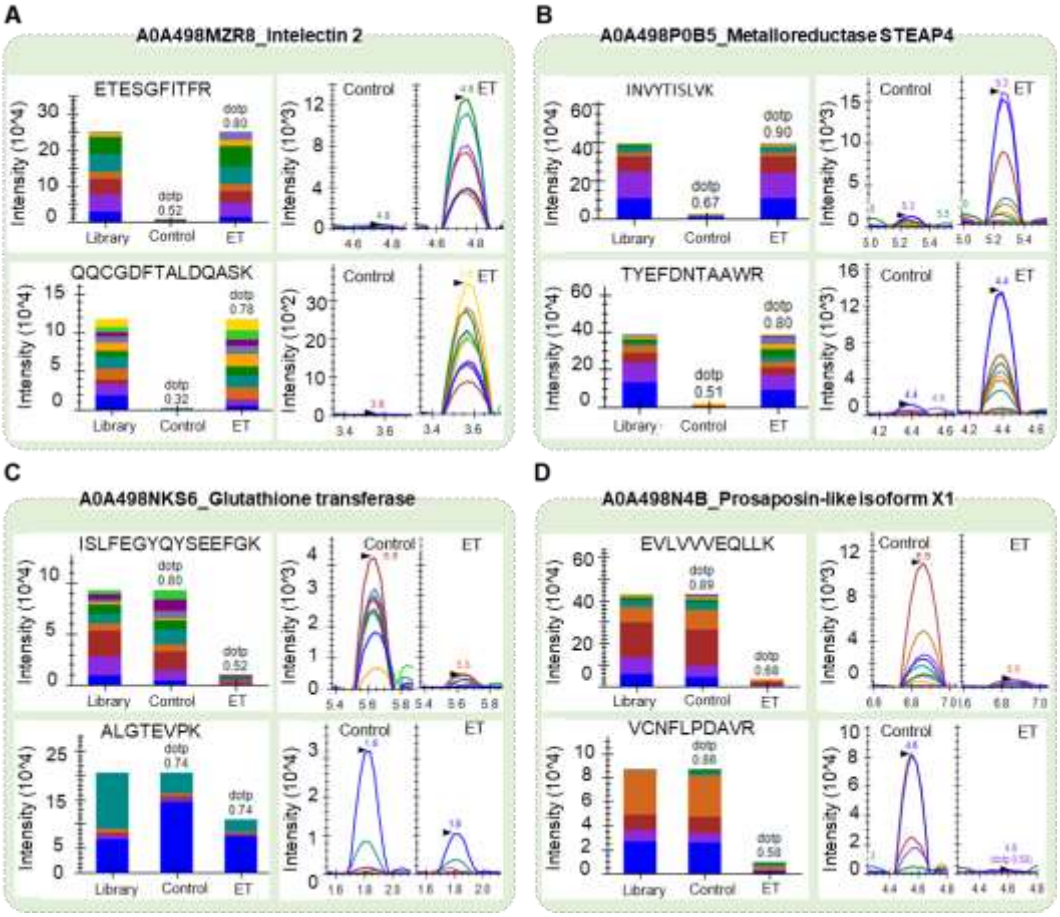
Figure 9. Landscape of altered pathways and interaction networks during *E. tarda* infection: Protein-Protein interaction of significant upregulated in the liver (**A)** and kidney (**B)** with respective KEGG and reactome pathways were represented (based on STRING different pathways highlighted in color).

4.8. Targeted proteomics of differentially expressed proteins across tissues

For targeted proteomics, data was acquired from DDA and analysed in Skyline software. A pooled sample run as quality control showed consistent responses from the instrument. Group comparison was performed between three Control (C1 to C3) and six ET (ET1 to ET3) samples. The analysis in liver ended up with 12 proteins, 32 peptides and 244 transitions. Of these, 28 peptides were found to be significant at different peak area between Control and ET group. Overall, the proteins showed a similar up/downregulation trend to that of discovery data indicating the validation of discovery proteomic trend. For Intelectin 2, five peptides were found to have significantly higher peak intensity in ET group compared to Control group (Fig. 10 (i)). For Metalloreductase STEAP4, Propionate-CoA ligase, D-aminoacyl-tRNA deacylase and Long-chain-fatty-acid-ligase 4, at least three peptides had significant peak intensity in ET group. Similarly, for Apolipo A-I-like protein, two peptides were significantly upregulated. Among downregulated proteins, Prosaposin had four significantly downregulated peptides in MRM data whereas Ricin B-type lectin domain-containing protein and Glutathione transferase showed only two peptides with significantly downregulated trend in ET group compared to Control.

In kidney, 3 peptides each of the 9 proteins, showing a fold change of >1.5 in ET as compared to Control (Fig. 10 (ii)). Out of these 4 proteins, showed 4-6 peptides were high-abundant in ET with a p value < 0.05 and a fold change value > 1.5, namely, Basement membrane specific Heparan proteoglycan core-like protein, Alpha 1 microglobulin, Mucin 2 like isoform X2 and Vitelline membrane outer layer 1-like protein. We also validated 3 proteins that were found to be low-abundant in the diseased condition.

i


A
A0A498MZR8_Intelectin 2
ETESGFITFR
dotp 0.52
dotp 0.80
Library
Control
ET
Intensity (10^4)
Intensity (10^3)
QQCGDFTALDQASK
dotp 0.32
dotp 0.78
Intensity (10^2)
B
A0A498P0B5_Metalloreductase STEAP4
INVYTISLVK
dotp 0.67
dotp 0.90
TYEFDNTAAWR
dotp 0.51
dotp 0.80
C
A0A498NKS6_Glutathione transferase
ISLFEGYQYSEEFGK
dotp 0.85
dotp 0.52
ALGTEVPK
dotp 0.74
dotp 0.74
D
A0A498N4B_Prosaposin-like isoform X1
EVLVVVEQLLK
dotp 0.89
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VCNFLPDAVR
dotp 0.86
dotp 0.58

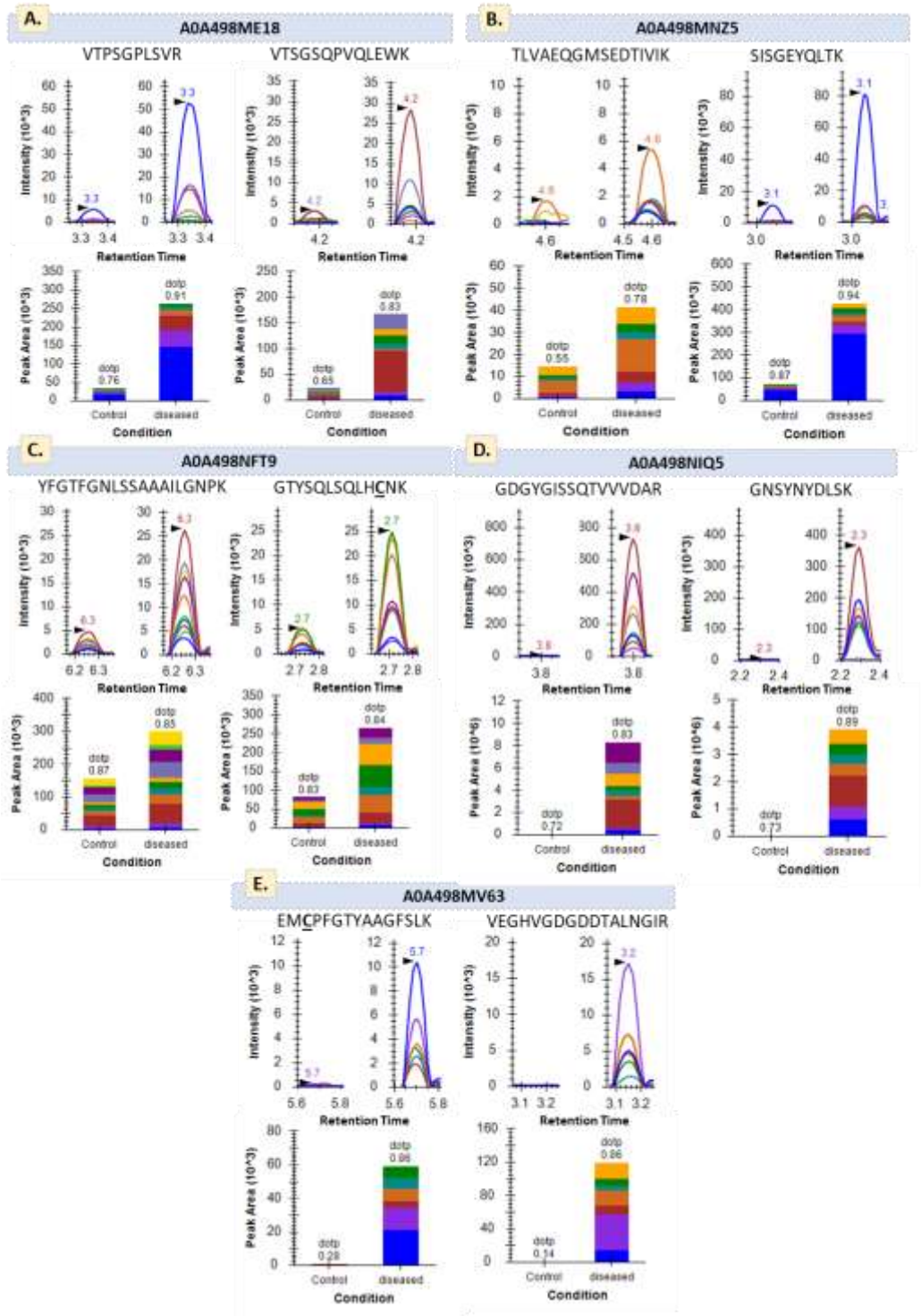

Figure 10. Validation for changes in protein abundance during *Et* infection using MRM: (i) in liver, (A-B) Peak area intensities and Peak shapes for two peptides each of proteins Intelectin 2 and STEAP4, respectively upregulated in *E. tarda* group compared to controls. (C-D) Peak area intensities and Peak shapes for two peptides each of proteins viz. Glutathione transferase and Prosaponin, respectively downregulated in *E. tarda* group. In each figure, left

panel shows the barplot representing the grouped peak area value for Control and *E. tarda* groups along with dot product (dotp) value obtained through spectral library. Right panel shows the representative MRM peak for respective peptide in both the groups. Different colors in the bar plots and spectral peaks represent different product ions of the same peptide. (ii) in kidney, each panel, two of the validated peptides are shown; peak shapes at the top followed by peak areas at the bottom. (A-B) Basement membrane-specific heparan sulfate proteoglycan core-like protein (A0A498ME18); Alpha-1-microglobulin (A0A498MNZ5); (C, D-E) Nitrogen permease regulator 3 isoform X2 (A0A498NFT9); Mucin-2-like isoform X2 (A0A498NIQ5); Vitelline membrane outer layer 1-like protein (A0A498MV63).

5. Summary

In the present study, the lethal dose of *Edwardsiella tarda* in rohu was 1.6 x 10^7 CFU/ml, with a mortality range of 58%. A combined histopathology and protein dynamics analysis provided a bigger picture of *E. tarda* pathogenicity in the rohu tissue. Histopathological analysis was used for the infection in three immunological tissues after being challenged with *E. tarda* to examine the abnormal structure and pathogenicity. The changes in the tissue of rohu histology in *E. tarda* infection showed pigmented phagocytes in liver tissue which formed melanomacrophage centers and showed typical vacuole formation, enlarged hepatocyte or hypertrophy. The infected kidney showed vacuolization, and occlusion of the tubular lumen cloudy, swelling degeneration, and necrosis of tubular epithelium. In the intestine, goblet cells hyperplasia and degenerative epithelium were noticed for *E. tarda*.

We used the liver, kidney and gut to identify the proteome of the rohu infected with *Edwardsiella tarda.* Lysates were prepared using SDS along with lysis buffer for the kidney and liver, and later label-free quantification was performed for both tissues. In the case of the gut, we used tandem mass tags (TMT) for peptides and later eluted them using LC-MS/MS. A total of ~1200 proteins were identified across the kidney and liver, while gut 1600 proteins were detected after considering ≥ 2 unique peptides. We observed differentially expressed proteins (DEPs) for rohu tissues, such as in the kidney-96, liver-257, and gut-29 proteins after 48 h infected with *E. tarda*. Apart from DEPs, unique proteins were identified across the tissue; kidney (259) and liver (343) and gut (575). Further gene ontology, functional analysis and protein-protein interaction of the

DEPs across the tissues were obtained using eggNOG, Metascape, KEGG and STRING. The present study has revealed in-depth knowledge of *E. tarda* infection in rohu tissues.

To validate the expressed proteins, a targeted proteomics Multiple Reaction Monitoring assay was used for highly dysregulated proteins between the control and infected groups in the kidney and liver. Targeted validation in the current studies is required to fill the knowledge gaps in the disease mechanism of *E. tarda* for rohu. Bacterial survival in the host is an important mechanism of infection and in our study, metalloproteases were altered which has a role in host infection observed in the rohu kidney. Copper and iron homeostasis is important for cell immune function and inflammatory response, in our study, metalloreductase steap4 was identified in response to *E. tarda in* both kidney and liver. In the kidney, metalloendopeptidase (bmp1a), was found to be significantly downregulated for *E. tarda*. Lysosomal proteins psap was low in the liver and expressed in infected kidney as a promising target for *E. tarda.* During *E. tarda* infection, a2m1 provides protection against the invasion process and clearance of damaged cells and other debris from the body. The study found that the intestine rohu was associated with changes in a number of proteins in the process of cell proliferation and metabolism. More studies are needed to explore the roles of these gut proteins involved in immunity and defence against bacterial infection.

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